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SYMPOSIUM ON NUCLEIC ACIDS AND NUCLEOPROTEINS

BY INVITATION OF THE COMMITTEE ON ARRANGEMENTS OF THE AUTUMN MEETING

Read before the Academy, November 6, 1958

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CHAIRMAN'S INTRODUCTION

By WENDELL M. STANLEY

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This symposium on nucleic acids and nucleoproteins was arranged because the sensitive probing point of really significant research in several important areas of biology and medicine seems to be leading directly to these materials and there is a pressing need for a greater general appreciation of the importance of the nucleoproteins and nucleic acids. For a long time the genetic material of all living things has been regarded as nucleoprotein in nature. Then the viruses, the smallest structures possessing many of the properties of living agents, were found to be nucleoprotein in nature, with many viruses being comprised solely of nucleoprotein, and some of these being crystallizable. Some of the most potent anticancer and mutagenic agents have been found to be analogues of components of nucleic acids.

In the enzymatic synthesis *in vitro* of deoxyribonucleic acid it has been found necessary to have present in the system a small amount of nucleic acid, presumably to act as a primer and to provide the pattern for the newly synthesized nucleic acid.* Nucleoprotein particles from cells have been found to provide the structural framework for the addition of activated amino acids in the synthesis *in vitro* of peptides. Thus nucleoproteins and more probably the nucleic acids seem to be the key material in the transfer of genetic information from parent to progeny, in mutation and hence in the evolutionary process, in the transformation of a normal cell into a malignant cell, in nucleic acid synthesis, and in protein synthesis.

To some the symposium this morning may seem to be unduly concerned with work on tobacco mosaic virus, hence a word of explanation may be in order. Tobacco mosaic virus and its strains would appear to represent the materials par excellence for launching an experimental attack on the basic problems just mentioned. This viral nucleoprotein and its strains can be obtained without undue effort in gram lots in preparations that are almost completely homogeneous—chemically, physically, and genetically. These viral nucleoproteins are highly organized rods 15 by 300 mμ in size and are composed of 5 per cent ribose nucleic acid and 95 per cent protein. The latter is, in turn, composed of about 2,100 sub-units of about 18,000 molecular weight arranged in helical fashion, and the nucleic acid appears to be a single elongated molecule of about 2 million molecular weight intermeshed in a helical pattern within the protein. These components can be separated to yield a protein preparation and a nucleic acid preparation and the latter has been found to retain the characteristic virus infectivity. Under certain conditions the protein and nucleic acid components can be reconstituted to form the original rod 15 by 300 mμ in size possessing full virus activity.

The very important fact that inoculation of a susceptible host with the nucleic acid preparation alone induces the formation of additional molecules of its own kind as well as the formation of a highly specific protein with which it eventually combines offers a direct experimental approach to the synthesis of polynucleotides and polypeptides and their interrelationships. If the chemical and genetic significance of the general association of nucleic acid and protein is ever to be elucidated, the tobacco mosaic virus system is probably the least complex we are likely to find for its study. Already much is known about the sequence of the 164 amino acids which make up the sub-unit of the protein component of this virus, and it seems reasonable to expect that we will be able to write out the exact amino acid sequence of this structure in the near future.

It is already known that the different strains of tobacco mosaic virus contain protein components which differ. While the amino acid sequence that is characteristic of a given strain is not known in such great detail as obtains in the case of the parent tobacco mosaic virus, it is known, for example, that a sub-unit from a given strain may characteristically contain a different amino acid at a given location. Thus there is ample reason to believe that every viral strain has an amino acid sequence that is uniquely characteristic for that strain. Since nucleic acid preparations alone are infectious, it is also reasonable to suppose that the nucleic acid of every viral strain has a polynucleotide sequence that is characteristic of that strain and that in each case a given polynucleotide sequence governs the corresponding polypeptide sequence. This conception provides a unique opportunity to probe

into the possible point-to-point structural relationships that may exist between the polynucleotide and polypeptide during the synthetic processes within the cell. Furthermore analogues such as fluorouracil, a compound possessing antitumor activity, can be incorporated into tobacco mosaic virus nucleic acid to a substantial extent and any resulting biological effects as well as any effect on the amino acid sequence within the progeny nucleoprotein can be studied. Tobacco mosaic virus thus provides a most favorable experimental material for these very important and very basic studies. It is unfortunate that polynucleotide chemistry is not nearly so advanced as is polypeptide chemistry and that the eventual solution of problems involving polynucleotide sequence may be some years in the future. However, as you will learn this morning, the pattern has been set and in time we confidently may expect to know the polynucleotide sequence that is characteristic of a replicating system, and something concerning the detailed structural changes involved in mutation and whether or not these can be controlled or perhaps even directed. We may also expect to learn something about the aberration in polynucleotide sequence that most probably is responsible for the transformation of a normal cell into a malignant cell. I trust, therefore, that this morning you will listen to the papers, some of which may appear rather detailed, with this larger view in mind. Work of this type and its extensions into the future are destined to permeate all of biology and medicine and eventually solve some of the most perplexing problems facing mankind.

* The presentation which was made by Howard K. Schachman of work with Arthur Kornberg entitled "Studies on the Enzymatic Synthesis of Deoxyribonucleic Acid" is not included among the following papers but some of this work has already been published in the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES [**44**, 633–647 (1958)].

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*ULTRACENTRIFUGAL FRACTIONATION AND IRON DISTRIBUTION IN INFECTIOUS NUCLEATES FROM TOBACCO MOSAIC VIRUS**

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Although the infectious materials prepared from tobacco mosaic virus, TMV, by Fraenkel-Conrat, Singer, and Williams[1] and by Gierer and Schramm[2] are generally considered to be salts of ribonucleic acid or ribonucleates, there has been neither agreement as to the molecular weight of the infectious component nor satisfactory explanations provided for their relatively low infectivity. With respect to the first question the authors first mentioned found that the infectivity was not sedimented under conditions (105,000 × g for 2 hr at about 0°C) where sedimentation might be expected for a particle of the order of 2×10^6 molecular weight. Accordingly they estimated that the infectious nucleate was of the order of 2×10^5 in molecular weight. Gierer,[3] on the contrary, has suggested on the basis of sedimentation velocity and infectivity studies that infectivity was associated only with nucleate particles of the size present in the original TMV rods, namely about 2×10^6. Partial agreement with the latter conclusion has come from the work of Hop-

kins and Sinsheimer[4] on nucleates prepared by heat denaturation. Light scattering measurements on such preparations which are comparable in infectivity[5] to those prepared with sodium dodecyl sulfate, SDS-nucleate, and with phenol, phenol-nucleate, gave an average molecular weight of 1.7×10^6.

In experiments reported from our laboratory some fractionation of virus infectivity was obtained when either SDS- or phenol-nucleates were subjected to similar centrifugal forces as used by Fraenkel-Conrat *et al.* but in larger tubes.[6] The data are reproduced in Table 1 where it may be seen that significant differences in activity were found particularly for the "lower" and "sediment" fractions in the case of the SDS-nucleate sample and for the "upper" and "lower" fractions for the phenol-nucleate. Considerable opportunity was available both in the experiments of Fraenkel-Conrat *et al.* and in our own for the re-solution of all or a part of the sedimented nucleate during the stopping of the centrifuge and removal of the fractions. In more recent experiments we have attempted to avoid such re-solution by carrying out the centrifugation in the presence of an immobilizing bed of coarse carborundum (*ca.* 0.4 gm of 100 mesh per 10 ml polyethylene tube). During centrifugation the bed is tightly packed near the end of the tube, and a solid yet relatively porous layer for the trapping of any sedimented material is provided. After removal of the supernatant liquid the carborundum is readily dispersed with a glass stirring rod and the trapped material can be extracted with water or with 0.01 *M* phosphate buffer at pH 7. By comparing the optical densities and infectivities of the upper and lower supernatant liquids and the redissolved sediment after ultracentrifugation with that of the original nucleate solution used, it was possible to determine the distribution of the original nucleate between the three fractions and to compare their respective infectivities. We have been able to examine only a few nucleate samples so far by means of this technique and while we have run into some inconsistencies, the results have shown definite fractionation both of infectivity and of iron content.

TABLE 1

INFECTIVITIES OF RNA FRACTIONS SEPARATED BY ULTRACENTRIFUGATION

	SDS-Nucleate				Phenol-Nucleate			
Fraction*	Av No. Lesions per Half-Leaf RNA	Virus	No. of Leaves	Mean Specific Infectivity ± 95% Confidence Interval†	Av No. Lesions per Half-Leaf RNA	Virus	No. of Leaves	Mean Specific Infectivity ±95% Confidence Interval†
Original	39	34	22	1.6 ± 0.3	18	19	22	1.2 ± 0.3
Upper	32	45	12	1.2 ± 0.7	4	38	25	0.7 ± 0.2
Middle	..	..	..	...	6	32	20	1.3 ± 0.4
Lower	36	62	10	0.8 ± 0.2	23	31	22	1.7 ± 0.4
Sediment	86	70	10	1.6 ± 0.3	33	52	8	0.7 ± 0.2

* The SDS-nucleate, 2.5 mg per ml, was centrifuged in a 10-ml polyethylene tube and 5 ml of "upper" and 4 ml of "lower" removed. The sediment was dissolved in 5 ml of 0.002 *M* phosphate. The respective concentrations found in milligrams per milliliter were 1.9, 3.1, and 0.4. The phenol-nucleate, 3 mg per ml, was centrifuged in a 5-ml polyethylene tube and 2 ml of "upper," 2 ml of "middle," and 1 ml of the "lower" fractions were removed. The sediment was redissolved in 2 ml of buffer. The respective nucleate concentrations found in milligrams per milliliter were 0.5, 0.5, 2.7, and 1.5.

† Result of two sample *t* tests at 95% confidence interval: "lower" fraction for SDS-nucleate significantly less infectious than either original or sediment. "Upper" fraction for phenol-nucleate significantly less infectious than either original or "lower" fraction.

The data shown in Table 2 illustrate the results found in three sedimentation experiments with one sample of SDS-nucleate and with two of phenol-nucleate. In the first two experiments the activity tests were made immediately after centrifugation and in the third after the respective fractions had been allowed to stand

in the cold for about 12 hr at the concentrations used for the activity tests. Data are recorded for the nucleate distribution between the three fractions, for the nucleate concentrations used for the infectivity tests, for the total numbers of lesions found for each fraction, and for the respective specific infectivities calculated as the weight per cent of virus which would be present in an otherwise noninfectious nucleate to account for the infectivity found.

TABLE 2

ULTRACENTRIFUGAL FRACTIONATION OF VIRUS INFECTIVITY IN PREPARATIONS OF SDS- AND PHENOL-NUCLEATES

Preparation and Concentration Centrifuged	Nucleate Distribution, %	Concentration Tested, %	Total No. of Lesions* Nucleate	Total No. of Lesions* Standard	Specific Infectivity, %
SDS, 0.3%†		0.024	184	206	1.3
Upper	47	0.024	125	745	0.2
Lower	50	0.024	212	677	0.5
Sediment	3	0.012	857	304	8.4
Phenol, 0.45%					
Upper	44	0.13	1,160	315	0.8
Sediment	8	0.002	212	531	6.2
		0.02	894	577	2.4
Phenol, 0.14%		0.024	1,722	1,018	2.1
Upper	47	0.1	2,050	801	1.0
Sediment	6	0.001	32	487	1.7

* Infectivity estimated by half-leaf method on 10 to 14 half-leaves of *N. glutinosa* in comparison with standard containing 0.003 mg of purified virus per milliliter. Infectivity tested immediately after centrifugation in the first two experiments and after standing for 12 hr in the third.

† In a similar experiment with another SDS-nucleate preparation the original sample gave an initial specific activity of 1.5% when compared at 10 optical density units (258 mμ) per milliliter with a virus standard at 0.01 optical density unit (265 mμ) per milliliter. After centrifugation as described in the text the "upper" fraction at the same nucleate concentration gave a specific activity of 0.8%. The sediment fraction at 3 optical density units per milliliter in comparison with a virus standard at 0.05 optical density unit per milliliter gave a specific activity of 8%.

It is clear from the experiment with the sample of SDS-nucleate, which has so far yielded the most striking results, that the initial activity of about 1.3 per cent was due to a relatively highly infectious component representing about 3 per cent or less of the total original nucleate. Because the sediment fraction contained a significant amount of the relatively noninfectious supernatant liquid, and because it produced about three times as many lesions as the standard virus, its percentage specific infectivity is probably two or three times higher than the value shown. Similarly the infectivities of the upper and lower supernatant liquid fractions are probably only about one-fifth the values shown.

The activity data for the second experiment with a sample of phenol-nucleate at a relatively high nucleate concentration unfortunately were not so conclusive as for the SDS-nucleate. Here it is not so clear that sedimentation of the infectivity was achieved but it should be pointed out that the nucleate concentration centrifuged was about 0.45 per cent as compared to about 0.3 per cent for the SDS-nucleate. It seems likely that at the higher nucleate concentration, possibly because of increased viscosity, the more infectious component was not so successfully sedimented.

If we accept the qualification mentioned for the phenol-nucleate experiment, the results are in agreement with our previous data that the SDS- and phenol-nucleates are inhomogeneous and consist of more infectious and either less infectious or noninfectious components. They are also in general agreement with the conclusions

reached previously by Gierer.[3] Because specific activities differing by several fold were found under comparable conditions, it is clear in answer to the second question raised in the beginning that the original low infectivity is not explained by the inability of the unprotected or "naked" nucleate to enter susceptible cells and to initiate the infection process as suggested originally by Fraenkel-Conrat and associates.[1] The possibility that the higher infectivities are due to small amounts of stable reconstituted virus is probably also eliminated because in the third experiment shown in which the infectivity of the sediment was determined after it had been allowed to stand in the cold for about 12 hr, only a small amount of activity, probably even less than that of the original sample was found. The high specific infectivity of the "sediment" appears, therefore, to be relatively unstable to dilution unlike that of the virus itself.

Of significance also in relation to possible reconstitution were the activity results found with the nonsedimenting components. Whereas the upper supernatant liquid was relatively low in activity, as for the SDS-nucleate which had been tested immediately after ultracentrifugation, it was comparable to the original phenol-nucleate in the third experiment after it had been allowed to stand in the refrigerator for about 12 hr. It thus appears that the more infectious component removed during ultracentrifugation was again reformed when the supernatant liquid was allowed to stand. The results mentioned may also explain not only the variation found in some of our own experiments but also the results reported by Fraenkel-Conrat and associates[1] in which no differences in specific activities were found in similar centrifugation experiments carried out, however, in the absence of an immobilizing bed.

Accompanying the differences in infectivity are differences in absorption characteristics as shown by the absorption spectra in 0.002 *M* phosphate at pH 7 for the upper, lower, and sediment fractions in the case of an SDS-nucleate preparation as reproduced in Figure 1. The more infectious "sediment" gave maximum to minimum ratios of about 1.4 as compared to values in the range 2.5 to 2.7 for the supernatant liquid. Of interest also was the fact that its absorbance per mole phosphorus per liter, 11×10^3, was about 12 per cent higher than either the original or the supernatant fractions.

From the original reconstitution experiments of Fraenkel- Conrat and Williams[7] and the more recent data of Takahashi,[8] it is now quite clear that the virus protein, under proper conditions, may recombine with a nucleate component present in preparations of SDS-or phenol-nucleates to form virus rods comparable in size and infectivity to those isolated by ultracentrifugation of infectious plant juice. From the present experiments and those of Gierer,[3] it is probable that virus protein combines with the more infectious nucleate component present in the SDS- or phenol-nucleate preparations, which in turn are in equilibrium with smaller noninfectious units. On this basis one may tentatively write two word equations for the reactions involved in virus reconstitution, namely

$$\text{tobacco mosaic virus protein} + \text{infectious nucleate} \rightleftharpoons \text{tobacco mosaic virus rods} \quad (1)$$

and

$$\text{noninfectious nucleate} + X \text{ (unknown)} \rightleftharpoons \text{infectious nucleate.} \quad (2)$$

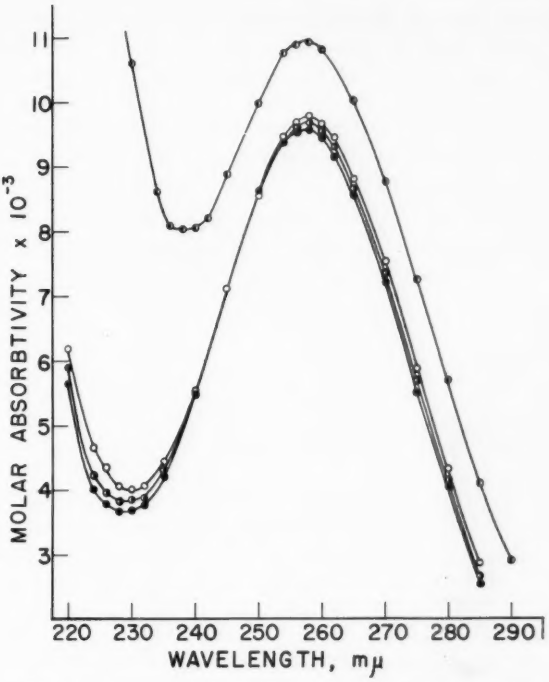

FIG. 1.—Absorption spectra per mole phosphate of fractions separated by ultracentrifugation of an aqueous solution of SDS-nucleate: ○, nucleate before ultracentrifugation; ●, upper supernatant fraction; ◑, lower supernatant fraction; ◐, "sediment" fraction.

We have included an unknown component in the second reaction particularly because of the occurrence of iron, calcium, magnesium, and probably copper and aluminum[6, 9, 10] in purified virus preparations in some tightly bound form.

Time is not available to review all of the evidence concerned, but our most recent data in the case of iron have shown that an amount corresponding to 1.5 mg per 100 gm or 10 to 11 atoms per mole occurs in different purified virus preparations with sufficient regularity to give a 95 per cent confidence interval of ± 0.2.[6] In experiments just completed we have also found radioactive iron, Fe^{59}, to be incorporated in much larger amounts in the isolated virus under conditions of rapid virus synthesis in newly infected Turkish tobacco plants (about 84 counts per minute per unit absorbance at 265 mμ) than under conditions prevailing in plants of the same age that had been infected for several weeks (about 3 counts per minute). We have prepared and fractionated the corresponding phenol-nucleates from such radioactive virus samples by high-speed centrifugation as described above. The data as summarized in Table 3 show: (1) that only about 5 per cent of the radioactive iron present in the virus was recovered in the nucleate isolated after phenol extraction and (2) that approximately three times as much of the radioactive iron remaining in the nucleate fraction per nucleate unit was present in the

sedimented as compared to the nonsedimented nucleate. Unfortunately, we do not have infectivity data on these particular fractions. The results, however, provide direct evidence that iron is at least one of the unknown components involved in the second equation mentioned. The large losses of iron in the phenol fractions during extraction of the virus with phenol are, in fact, better correlated with the large losses of infectivity accompanying this treatment than is the loss of ribonucleate itself.

TABLE 3

Fe^{59} DISTRIBUTION IN VIRUS, IN PHENOL-NUCLEATE, AND IN FRACTIONS SEPARATED IN PRESENCE OF IMMOBILIZING LAYER

Sample	Total Absorbance	Total Counts per Minute	Counts / Absorbance
TMV	1,723	2,800	1.6
Phenol–nucleate	438	149	0.34
Upper	139	55	0.39
Lower	209	61	0.29
Sediment	30	33	1.1

Absorbance measurements for the virus and nucleate fractions taken at 265 and 258 mμ, respectively.

Summary.—In summary it may be said that a new ultracentrifugation technique, namely the use of an immobilizing carborundum bed, is described for the fractionation of sedimentable but highly diffusible materials. By the use of this technique new evidence has been provided for the presence of a more highly infectious, sedimentable, nucleate component in preparations obtained by treating tobacco mosaic virus with sodium dodecyl sulfate or phenol. As the infectivity of the highly active component in the sediment fraction soon decreases and that of the lowly active component in the supernatant liquid increases to about the original level when the infective solutions are allowed to stand, the concentration of the more highly infectious component appears to be the result of a dynamic equilibrium between infectious and noninfectious nucleate components.

Radioactive iron, Fe^{59}, was incorporated into the virus during its rapid synthesis in newly infected Turkish tobacco plants in about 25 times the quantity found when the same amount of radioactive iron was administered to fully infected plants.

When the radioactive virus was treated with phenol for the preparation of the nucleate fraction, about 95 per cent of the Fe^{59} was removed together with protein in the phenol extracts. When the radioactive nucleate fraction was subject to ultracentrifugation in the presence of an immobilizing carborundum bed, iron was concentrated in the more highly infectious sediment in roughly the same ratio as the increase in infectivity.

Note added in proof (Feb. 20, 1959): In two additional centrifugation experiments with phenol-nucleate preparations at a concentration of 0.3 per cent, the infectivities of the "upper" and "sediment" fractions were tested under conditions where comparable numbers of lesions were found for each fraction in comparison with the virus standard. Calculation of specific activities showed that the "sediment" fraction was two to three times as infectious as the "upper" fraction when the samples were inoculated immediately after ultracentrifugation. The differences likewise proved to be statistically significant at the 99.9 per cent confidence level. When the respective infectivities were compared 24 hr later, apparent increases were found in the "upper" fractions and apparent decreases in the "sediment" fractions. The differences in specific activities between the two fractions after 24 hr were not statistically significant at the 95 per cent confidence level.

* Aided by research grants from the American Cancer Society (Grants No. E-31 and E-128) and the National Heart Institute, United States Public Health Service (Grant No. H 1938).

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*THE ARRANGEMENT OF PURINE AND PYRIMIDINE NUCLEOTIDES IN TOBACCO MOSAIC VIRUS NUCLEIC ACID**

BY K. K. REDDI

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The nucleic acid in tobacco mosaic virus (TMV) constitutes 5 per cent of the virus.[1] It is a highly polymerized molecule built up from three types of chemical entities: (1) nitrogen containing bases, the purines and pyrimidines, (2) a pentose, ribose, and (3) phosphoric acid. It has a characteristic base composition and the four bases, adenine, guanine, cytosine, and uracil occur in dissimilar proportions.[2] Recent results indicate that the molecular weight of this nucleic acid is about 2×10^{6},[3] hence it is made up of about 6,000 nucleotides. As yet little evidence has been obtained concerning the sequential arrangement of these nucleotide residues. The arrangement is, however, most likely not random. The information relating to the arrangement of nucleotides in a nucleic acid molecule could be obtained by using a suitable stepwise degradation procedure. Unfortunately no such procedure is available at present. The method proposed by Brown, Fried, and Todd[4] has not so far been used to determine the sequences in polynucleotides bigger than di- and trinucleotides.[5] Some idea of the arrangement of the individual nuleotide residues may, however, be gained by the use of nucleases of known specificity. One such nuclease which has been used with great advantage is pancreatic ribonuclease which hydrolyzes only pyrimidine ribonucleoside 3′-phosphate linkages.[6, 7, 8] By investigating the breakdown products formed by its action it is possible to obtain an idea of the arrangement of some of the nucleotide residues in a nucleic acid molecule. The information presented in this paper concerning the arrangement of some of the purine and pyrimidine nucleotides in tobacco mosaic virus nucleic acid was obtained by using pancreatic ribonuclease and also to some extent the micrococcal phosphodiesterase.

Isolation and Analysis of Purine Rich Oligonucleotide Fragments in Pancreatic Ribonuclease Digests of Tobacco Mosaic Virus Nucleic Acid.—The tobacco mosaic virus nucleic acid (TMV-NA) was digested exhaustively with pancreatic ribonuclease and the ribonuclease resistant residues were separated into fractions, soluble and

insoluble, respectively, in 6 per cent trichloroacetic acid[9] (TCA). About 14 per cent of the original nucleic acid was present in the insoluble fraction and this was rich in purines and poor in pyrimidines. The content of adenine was higher than that of any other base. From the base composition and the specificity of pancreatic ribonuclease it could be suggested that the average size of this residual fraction is six nucleotides in length (5 purine nucleotides terminated by a pyrimidine nucleotide). The sedimentation constant of 0.9 *S* of this material is consistent with this size. Thus, there is evidence to show that in TMV-NA some of the purine nucleotide residues occur clustered as polypurine nucleotide segments flanked by pyrimidine nucleotides. The amounts of adenylic acid and guanylic acid residues that occur in this manner were calculated using the base composition data for these oligonucleotide segments. It was found that 21.6 per cent of total adenylic acid residues and 20.4 per cent of total guanylic acid residues occur in TMV-NA as polypurine nucleotide structures insoluble in 6 per cent TCA (Tables 1 and 2).

TABLE 1

MODE OF ARRANGEMENT OF SOME ADENYLIC ACID RESIDUES IN TMV-NA AS REVEALED BY DIGESTION WITH PANCREATIC RIBONUCLEASE

Nature of Compound	Sequence	Calculated as Per Cent of Total Adenylic Acid Residues in TMV-NA
ApCp	PypApCp	7.0
ApUp	PypApUp	8.1
ApGpCp	PypApGpCp	18.5
Purine nucleotide clusters*	PypPup---PupPyp	21.6

* These represent 6% TCA insoluble fraction in pancreatic ribonuclease digests of TMV-NA, having an average size of 6 nucleotides: 5 purine nucleotides (adenylic and guanylic acids) terminated by a pyrimidine nucleotide.

TABLE 2

MODE OF ARRANGEMENT OF SOME GUANYLIC ACID RESIDUES IN TMV-NA AS REVEALED BY DIGESTION WITH PANCREATIC RIBONUCLEASE

Nature of Compound	Sequence	Calculated as Per Cent of Total Guanylic Acid Residues in TMV-NA
GpUp	PypGpUp	9.3
ApGpCp	PypApGpCp	11.1
Purine nucleotide clusters*	PypPup---PupPyp	20.4

* These represent 6% TCA insoluble fraction in pancreatic ribonuclease digests of TMV-NA, having an average size of 6 nucleotides: 5 purine nucleotides (adenylic and guanylic acids) terminated by a pyrimidine nucleotide.

Isolation and Estimation of Monopyrimidine Nucleotides in Pancreatic Ribonuclease Digests of TMV-NA.—When TMV-NA was digested with pancreatic ribonuclease there was a gradual appearance of cytidylic and uridylic acids in the digest with time[10] and this attained a maximum by 12 hrs and remained so even after digestion for 18 hrs. About 50.7 per cent of total uridylic acid residues and 45.7 per cent of total cytidylic acid residues present in the intact TMV-NA were released as monopyrimidine nucleotides in 12 hrs. For a monopyrimidine nucleotide to appear in a pancreatic ribonuclease digest of a ribonucleic acid, the minimum requirement is the occurrence of at least two pyrimidine nucleotides linked together. It is evident from these results that about 50 per cent of total pyrimidine nucleotide residues in TMV-NA occur in "clusters" of two or more pyrimidine nucleotides.

Isolation and Estimation of Di- and Tri-nucleotides in Pancreatic Ribonuclease Digests of TMV-NA.—A complete digest of TMV-NA was examined for the presence of di-nucleotides. Only three di-nucleotides, ApCp, ApUp, and GpUp,† were found[11] and the amounts of these are given in Tables 1, 2, 3, and 4. These data and the specificity of pancreatic ribonuclease permit certain conclusions to be drawn concerning the arrangement of some of the nucleotide residues in TMV-NA. For di-nucleotides, ApCp, ApUp, and GpUp to appear in a pancreatic ribonuclease digest of TMV-NA, the nucleotides, adenylic acid, and guanylic acid must be preceded by a pyrimidine nucleotide. The sequence of the type PypApCpRp, PypApUpRp, and PypGpUpRp (where Pyp is a pyrimidine nucleotide, cytidylic or uridylic acid and Rp is any one of four nucleotides) exist in TMV-NA. The sequence of the type PypGpCpRp must not be present since the di-nucleotide, GpCp, is not present in the complete digest of TMV-NA. Instead, a sequence of

TABLE 3

MODE OF ARRANGEMENT OF SOME CYTIDYLIC ACID RESIDUES IN TMV-NA AS REVEALED BY DIGESTION WITH PANCREATIC RIBONUCLEASE

Nature of Compound	Sequence	Calculated as Per Cent of Total Cytidylic Acid Residues in TMV-NA
ApCp	PypApCp	11.3
ApGpCp	PypApGpCp	15.9
Terminal groups of purine nucleotide clusters (Tables 1 and 2)	Pup---PupCp	5.0
Pyrimidine nucleotide clusters*	PupPypPyp---Pup	45.7

* Pyrimidine nucleotide clusters containing two or more pyrimidine nucleotides flanked by purine nucleotides.

TABLE 4

MODE OF ARRANGEMENT OF SOME URIDYLIC ACID RESIDUES IN TMV-NA AS REVEALED BY DIGESTION WITH PANCREATIC RIBONUCLEASE

Nature of Compound	Sequence	Calculated as Per Cent of Total Uridylic Acid Residues in TMV-NA
ApUp	PypApUp	8.1
GpUp	PypGpUp	8.4
Terminal groups of purine nucleotide clusters (Tables 1 and 2)	Pup---PupUp	5.1
Pyrimidine nucleotide clusters*	PupPypPyp---Pup	50.7

* Pyrimidine nucleotide clusters containing two or more pyrimidine nucleotides flanked by purine nucleotides.

the type PypApGpCpRp is present since a tri-nucleotide, ApGpCp (one tri-nucleotide was so far isolated) is present in the digest. Thus guanylic acid preceded by a pyrimidine nucleotide and followed by a cytidylic acid appears not to exist in TMV-NA.

A summary of the mode of arrangement of some of the nucleotide residues in TMV-NA as revealed by digestion with pancreatic ribonuclease is given in Tables 1, 2, 3, and 4. The information so far obtained concerning the mode of arrangement of nucleotide residues in TMV-NA, accounts for 55.2, 40.8, 77.9, and 72.3 per cent of the total adenylic acid, guanylic acid, cytidylic acid, and uridylic acid residues respectively in TMV-NA. These results show that in addition to purine

and pyrimidine nucleotides occurring in clusters of two or more, they also exist singly alternating with each other. However, the former type of arrangement appears to be more predominant in TMV-NA than the latter type. There is a certain regularity in their arrangement. Thus, for instance, of the total adenylic acid and guanylic acid residues about 20 to 22 per cent of each occur as polypurine nucleotide segments (6 per cent TCA insoluble). Of the total cytidylic and uridylic acid residues about 50 per cent of each occur as polypyrimidine nucleotide segments and 5 per cent of each as terminal groups for polypurine nucleotide segments (6 per cent TCA insoluble). Thus there is a definite regularity in the way the purine nucleotides and the pyrimidine nucleotides are arranged in TMV-NA and this excludes the possibility of randomness.

Isolation and Estimation of Di-nucleotides in the Micrococcal Phosphodiesterase Digests of TMV-NA.—Recently Cunningham, Catlin, and de Garilhe[13] reported the presence of a calcium activated nuclease in the culture medium of *Micrococcus pyogenes* var. *aureus*. They have partially purified this enzyme and have studied its action on deoxyribonucleic acid. This nuclease was also found to hydrolyze ribonucleic acid.[14] This enzyme has been purified in our laboratory and its action on TMV-NA was studied.[15] In the complete digest several di-nucleotides in addition to mononucleotides, adenosine 3′-phosphate, guanosine 3′-phosphate, cytidine 3′-phosphate and uridine 3′-phosphate were found.‡ In Table 5 are

TABLE 5

DI-NUCLEOTIDES IN MICROCOCCAL PHOSPHODIESTERASE DIGEST OF TMV-NA

Di-nucleotides	Per Cent Total Adenylic Acid in TMV-NA	Per Cent Total Guanylic Acid in TMV-NA	Per Cent Total Cytidylic Acid in TMV-NA	Per Cent Total Uridylic Acid in TMV-NA
ApCp	10.0	...	16.2	...
ApUp	11.4	...	...	11.5
ApGp	17.6	19.7	...	...
ApAp	8.4	...	...	...
UpGp	...	19.7	...	17.7
UpCp	...	...	12.3	7.7
UpUp	...	...	...	13.1

TABLE 6

DI-NUCLEOTIDE SEQUENCES IN A POLYRIBONUCLEOTIDE MADE UP OF ADENYLIC, GUANYLIC, CYTIDYLIC, AND URIDYLIC ACID RESIDUES

ApAp	GpGp	CpCp
ApCp	*GpCp*	*UpUp*
CpAp	CpGp	CpUp
ApUp	*GpUp*	*UpCp*
UpAp	*UpGp*	...
ApGp	...	...
GpAp	..	...

Those italicized have been so far found in the pancreatic ribonuclease and micrococcal phosphodiesterase digests of TMV-NA.

listed the di-nucleotides and their amounts. The action of this enzyme on TMV-NA reveals some new types of di-nucleotide sequences, such as UpCp, ApAp, UpGp, and UpUp. However, the data do not permit assignment of the exact specificity for this enzyme.

In TMV-NA containing adenylic, guanylic, cytidylic, and uridylic acids 16 different di-nucleotide sequences are possible (Table 6). Of these 16 those itali-

cized in Table 6 have been so far found in the pancreatic ribonuclease and micrococcal phosphodiesterase digests of TMV-NA. Using specific nucleases different from the ones so far used it should be possible to find the remaining di-nucleotide sequences if these are present in TMV-NA.

Structural differences in TMV-strain nucleic acids: The above approach at revealing the arrangement of purine and pyrimidine nucleotides in TMV-NA by degrading it with a specific nuclease is also useful in differentiating nucleic acids which are closely allied and have similar base composition. Tobacco mosaic virus is well known for its numerous strains or mutants which exhibit different biological symptoms and yet their nucleic acids have similar purine and pyrimidine composition.[16, 17] Recent experiments of Fraenkel-Conrat[18] and Gierer and Schramm[19] show that nucleic acid in TMV is a fundamental genetic unit. If the nucleic acid represents the genetic material of a virus it would seem that nucleic acids of viral strains must differ in at least some structural features. This possibility has been examined.

Isolation and Analysis of Purine Rich Oligonucleotide Fragments in Pancreatic Ribonuclease Digests of Tobacco Mosaic Virus Strain Nucleic Acids.—The nucleic acids obtained from five strains of tobacco mosaic virus were maximally digested with pancreatic ribonuclease. The ribonuclease resistant residues which are precipitable by 6 per cent TCA were analyzed for their purine and pyrimidine composition[9] and they were found to have the same composition. These results indicate some structural similarity in the nucleic acids of different strains and also show that in all strain nucleic acids some of the purine nucleotide residues tend to cluster and the over-all composition of such clusters is essentially the same. However, the amounts§ of these purine nucleotide clusters and also the sequence of adenylic and guanylic acid residues in them might be different in each case. This possibility is being examined at present.

Differences in the Intramolecular Distribution of Pyrimidine Nucleotides in Tobacco Mosaic Virus Strain Nucleic Acids.—Although there is no difference in the base composition of the strain nucleic acids and their pancreatic ribonuclease resistant residues (6 per cent TCA insoluble), it is probable that the way the individual nucleotides are arranged in each case might be different. Hence it was thought an examination of pancreatic ribonuclease digests might reveal some differences in

TABLE 7

MONOPYRIMIDINE NUCLEOTIDES, CYTIDYLIC, AND URIDYLIC ACIDS IN THE PANCREATIC RIBONUCLEASE DIGESTS OF TMV STRAIN NUCLEIC ACIDS

	Cytidylic Acid (Cp)		Uridylic Acid (up)	
Strain	γ/mg NA	Per Cent Total Cp in NA	γ/mg NA	Per Cent Total Up in NA
TMV	79.9	45.7	127.5	50.7
HR	73.5	42.0	120.8	48.1
YA	78.8	45.0	124.0	49.3
M	107.1	61.2	178.1	70.8

the intramolecular distribution of pyrimidine nucleotides in the nucleic acids of TMV strains. The nucleic acids obtained from four strains of TMV were maximally digested with pancreatic ribonuclease and the monopyrimidine nucleotides, cytidylic, and uridylic acids present in each of the digests were estimated.[10] The amounts of monopyrimidine nucleotides present in the digests of TMV, HR,

and YA strain nucleic acids were the same while in the digest of M strain nucleic acid there was a significant difference (Table 7). Since the base composition of all the strain nucleic acids is the same, the greater amounts of monopyrimidine nucleotides in M strain nucleic acid can be attributed only to the difference in the arrangement of pyrimidine nucleotides. The appearance of larger amounts of monopyrimidine nucleotides in M strain nucleic acid digest is a clear indication of the occurrence of a larger proportion of pyrimidine polynucleotide segments than in the nucleic acids of TMV, HR, and YA. Thus the intramolecular distribution of pyrimidine nucleotides in M strain nucleic acid differs from those of strains TMV, HR, and YA. The polypyrimidine nucleotide segments in the nucleic acids of TMV, HR, and YA might still differ in the way the cytidylic and uridylic acids are arranged in them. This possibility can be examined by using a nuclease having specificity exactly opposite to that of pancreatic ribonuclease.

Di- and Tri-nucleotides in the Pancreatic Ribonuclease Digests of Tobacco Mosaic Virus Strain Nucleic Acids.—The nucleic acids obtained from TMV, HR, and M were digested exhaustively with pancreatic ribonuclease. In the digests of nucleic acids of TMV, HR, and M only three di-nucleotides, ApCp, ApUp, and GpUp, were found. There was no di-nucleotide GpCp in either of these digests. One tri-nucleotide ApGpCp was also isolated from these digests.[11] However, the amounts of di-nucleotides and also tri-nucleotide found in the digests of nucleic acids obtained from these strains differed significantly (Tables 8 and 9). The amounts of

TABLE 8

DI-NUCLEOTIDES IN THE PANCREATIC RIBONUCLEASE DIGESTS OF TMV STRAIN NUCLEIC ACIDS

Strain	ApCp, γ/mg NA	ApUp, γ/mg NA	GpUp, γ/mg NA
TMV	39.4	44.8	47.6
HR	35.6	40.8	55.1
M	44.3	46.8	48.9

TABLE 9

TRI-NUCLEOTIDE, ADENYLYL-GUANYLYL-CYTIDYLIC ACID IN PANCREATIC RIBONUCLEASE DIGESTS OF TMV STRAIN NUCLEIC ACIDS

Strain	ApGpCp, γ/mg NA
TMV	85.3
HR	72.9
M	72.6

ApCp present in the digests of nucleic acids of TMV and HR differed significantly from the amount present in the digest of M strain nucleic acid. The amounts of GpUp and ApUp present in the digests of TMV and M strain nucleic acids were the same and differed significantly from the amounts present in the digest of HR nucleic acid. The same amounts of ApGpCp were found in the digests of HR and M strain nucleic acids while the amount present in TMV strain nucleic acid was significantly different. Since the base composition of these three nucleic acids is the same, these differences in the amounts of di- and tri-nucleotides present in the pancreatic ribonuclease digests can be attributed only to differences in the way the nucleotides are distributed in each of the nucleic acids of TMV, HR, and M. The

di- and tri-nucleotides appear in the pancreatic ribonuclease digest of a nucleic acid only if sequences of the type PypPupPyp and PypPupPupPyp are present in it. The amounts of these present in the digest depend upon the number of such groups present in the intact nucleic acid.

These results demonstrate that there are certain similarities and also significant differences in the sequential arrangement of the nucleotides in the nucleic acids of TMV, HR, and M. This constitutes the first evidence of structural differences in the nucleic acids of different strains of a virus and thus provides a chemical basis for the biological differences exhibited by these strains. Studies of this nature using nucleases of different specificity may reveal more information concerning the sequential arrangement of the nucleotides in the strain nucleic acids. A complete knowledge of these differences should lead to a better understanding of the nature of the mutation process and also should provide a rational approach for modifying the nature of a virus by reshuffling the arrangement of the nucleotides in its nucleic acid moiety.

Summary.—Information pertaining to the mode of arrangement of some of the purine and pyrimidine nucleotides in tobacco mosaic virus nucleic acid was obtained using nucleases such as pancreatic ribonuclease and micrococcal phosphodiesterase. These results show that in addition to both the purine nucleotides and pyrimidine nucleotides occurring in clusters of two or more, they also exist singly alternating with each other. The latter type of arrangement appears not to be so frequent as the former. There is a definite regularity in the arrangement of both the purine and pyrimidine nucleotides in TMV-NA, suggesting that the arrangement is not one of random type.

Examination of the products obtained by digestion of nucleic acids of TMV, HR, and M with pancreatic ribonuclease revealed significant differences in the mode of arrangement of purine and pyrimidine nucleotides in each of these molecules. This information provides evidence that the genetic specificity of a nucleic acid resides in the arrangement of its components, namely the purine and pyrimidine nucleotides. The importance of this finding on the phenomenon of mutation has been discussed.

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† Shorthand notation used to denote oligonucleotides is the one suggested by Heppel, Whitfield, and Markham.[12]

‡ A paper describing the details of the purification of this enzyme and also methods employed in isolating and identifying the products of degradation is in preparation.

§ Only in the case of TMV-NA the amount of 6 per cent TCA precipitable material in the pancreatic ribonuclease digest was determined and was found to be about 14 per cent of the original intact nucleic acid.[9]

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OBSERVATIONS ON THE BIOLOGICAL BEHAVIOR OF TOBACCO MOSAIC VIRUS NUCLEIC ACID

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While I am honored to participate in this symposium, I must point out that I am a spokesman for a group of close friends and associates located on the Los Angeles campus of the University of California. What I have to say is provided by the recent work of Dr. William Ginoza of the UCLA Atomic Energy Project, Professor Amos Norman of the Department of Radiology in the School of Medicine, and Dr. Albert Siegel of the Botany Department. The general objective of their experimentation is to acquire a greater understanding of the interaction of tobacco mosaic virus (TMV) with its host, *Nicotiana glutinosa*, a host in which TMV induces the formation of local lesions. Most of the experiments that I will discuss have been published so I will attempt only to summarize the findings.

Nearly all of the experiments revolve around the use and interpretation of dose-survivor curves. A treatment is applied to the virus, and the fraction of virus activity which survives the treatment is measured by local lesion bioassay as a function of the dose of the treatment. One killing dose is achieved when the biological activity of a population of virus particles is reduced to 37 per cent of that of the untreated material. If those particles which survived the first killing dose are again treated, a second killing dose is attained when the remaining activity is again reduced to the level of 37 per cent survivors, etc. The usual interpretation of such results is that with one killing dose, each virus particle, on the average, has suffered at least a single hit from the treatment; 2 hits with 2 doses, etc. The treatments that have been employed are X rays, ultraviolet light (UV), and heat.

Shortly after Fraenkel-Conrat[1] and Gierer and Schramm[2] discovered that the biological activity of the TMV particle was possessed by the nucleic acid, Ginoza and Norman[3] were prompted to investigate the size of nucleic acid essential for infectivity. Lauffer *et al.*[4] using X-ray inactivation of intact TMV had found that the sensitive volume of the virus particle was about one-twelfth of the total volume

of the virus rod. Since nucleic acid amounts to only 6 per cent of the total mass of the virus, they inferred that it was the nucleic acid which occupied the sensitive volume essential for the biological activity of the virus. When it was learned that nucleic acid could be extracted from the particle and still retain virus infectivity, Ginoza and Norman were able to determine by X-ray inactivation experiments that the sensitive volume of the extracted nucleic acid corresponded to an infectious material having a molecular weight in the magnitude of one or two million. Since on the basis of these experiments and different experiments from other laboratories, there now seems to be general agreement that nucleic acid of the order of one million molecular weight is essential for infectivity, I will not attempt to justify further this concept, but instead, I will turn to a discussion of Ginoza's notion that infectious TMV nucleic acid is a highly elongated, single-stranded structure. His conception is based largely on the inactivation of TMV nucleic acid by heat.[5]

The data plotted in Figure 1 are typical of the inactivation behavior of nucleic

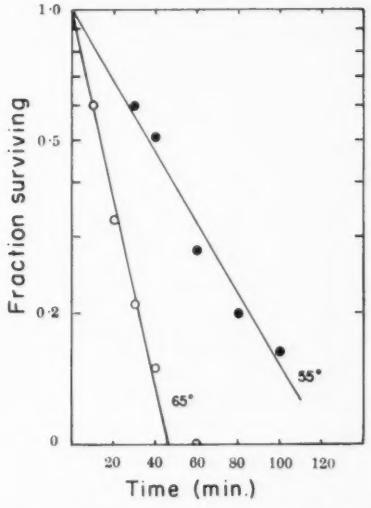


FIG. 1.—Survival curves of tobacco mosaic virus ribonucleic acid heated at pH 7.15 in 0.1 *M* phosphate buffer. From Ginoza, *Nature*, **181**, 958 (1958).

acid resulting from heating the material for various lengths of time and at different temperatures. An exponential decrease in biological activity is observed as a function of the time of heating. From the graph, 58 min of heating at 55° reduces the virus activity to 37 per cent of that of the untreated material, which is equivalent to one killing dose of heat. Continuing the heat treatment for another 58 min results in two doses, etc. If we suppose that the effect of heat is to break, at random, some of the 6,000 phosphodiester linkages joining the nucleotides together into a threadlike molecule of nucleic acid, then we can imagine that with one dose of heat, each molecule of nucleic acid in the population which possesses viricidal activity would have suffered at least one break in the chain; two breaks with two doses, etc. The exponential character of the heat inactivation curve is interpreted to signify that a single break of any of the 6,000 linkages is sufficient to render the molecule inactive as a virus. Ginoza has sought for other experimental evidence to support the hypothesis that heat breaks phosphodiester bonds.

The apparent ratio of viricidal nucleic acid to inactive nucleic acid is about 1 to 100. If the inactive material behaves in the same way in regard to heat as the active nucleic acid, then the bulk of the preparation should display predictable changes in properties such as viscosity, sedimentation behavior, and the appearance of mono- and dinucleotides as a function of the number of heat doses. Ginoza has found, for example, that without heat treatment, most of the nucleic acid sediments in a gravitational field as a single component. Treatment with heat reduces the amount of this component and increases the amount of components which sediment more slowly, and hence, are of smaller size than the original material. While the results are qualitative, the distinct impression is gained that the amount of transformation is proportional to the number of killing doses, and therefore related to the number of breaks produced in the nucleic acid chains. The viscosity of TMV nucleic acid also decreases in accordance with the number of heat-killing doses applied, a result which is consistent with the sedimentation findings.

If heating TMV nucleic acid at 55° results in one break each 58 min, approximately 100 days of heating would be required to produce 3,000 breaks per molecule. Thus, application of 3,000 killing doses of heat should leave the preparation predominately in the form of mono- and dinucleotides. This prediction is borne out by Ginoza's observation that the amount of nucleotides being formed as the result of continued heating ceases to increase in amount at around the 100th day of incubation.

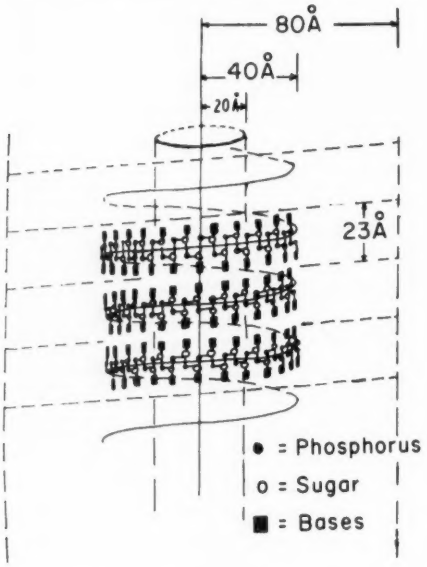


FIG. 2.—Schematic representation of a single-strand nucleic acid helix of 40-A radius in position within the tobacco mosaic virus rod. The spacings of the purine and pyrimidine bases, ribose rings, and phosphorus atoms with respect to the helix are made approximately to scale. There are about 50 bases per turn of the helix, aligned parallel to the longitudinal axis of the virus particle. A central hole of 20-A. radius extends the length of the particle. A protein matrix of 60-A. radius or thickness (80-A. radius of the virus less 20-A. radius of the hole) is composed of a few thousand equivalent subunits set in a helical array about the axis of the rod. From Ginoza, *Nature*, **181**, 958 (1958).

Thus, there is evidence in favor of Ginoza's concept of the TMV particle consisting of a threadlike nucleic acid molecule composed of 6,000 nucleotides being surrounded by protein subunits. Figure 2 is a schematic representation of a single strand of nucleic acid coiled within a tobacco mosaic rod. On the basis of X-ray

crystallographic evidence, Dr. Rosalind Franklin, shortly before her untimely death, came to a similar conclusion that TMV is fashioned out of a thread of nucleic acid locked in place by protein subunits,[6] and Figure 3 is a photograph of the beautiful model she constructed for display at the Brussels World Fair.

Gierer[11] has also come to the same conclusion in regard to the shape and size of infectious TMV nucleic acid as the result of degrading the material with ribonuclease. Both Gierer and Ginoza conclude, as the result of experiments with agents

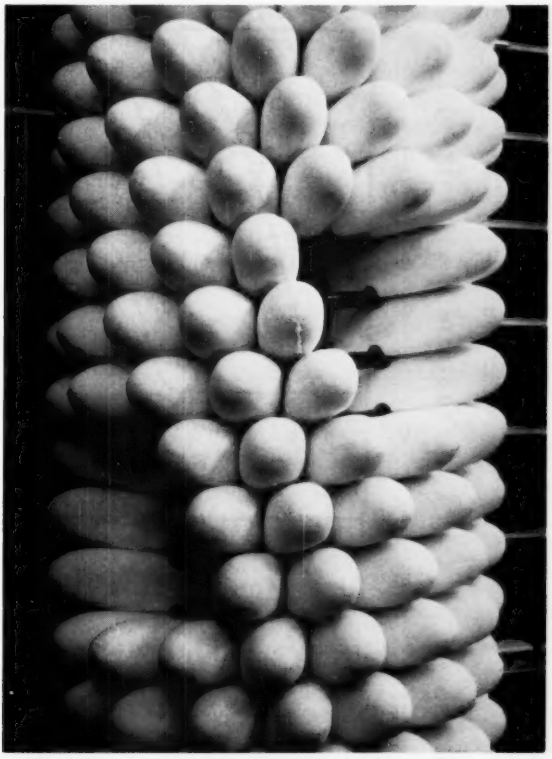


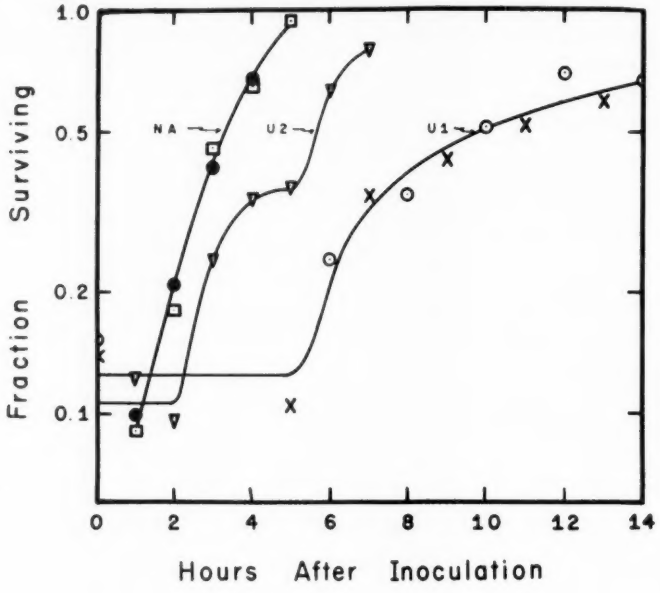
FIG. 3.—Photograph of model of TMV particle constructed by Dr. Rosalind Franklin showing entrapment of a single strand of nucleic acid (black) by closely packed protein subunits (white). Photo courtesy of Dr. Donald Caspar, Yale University.

which disrupt hydrogen bonds, that secondary bonding of the nucleic acid is not essential for the biological integrity of the material.

Dr. Albert Siegel has performed ultraviolet light experiments which provide information concerning the behavior of TMV during the earliest stages of its reproductive activity. The information has arrived from the interpretation of dose-survival curves employing the Luria-Latarjet[7] technique of inactivating the virus after it has made contact with the plant host cells. Both outside and inside the host, Siegel finds strains to differ markedly in their sensitivity to inactivation with UV.[8] One strain, U2, is $5^1/_2$ times more sensitive than the common strain, U1. The difference is connected with the presence of protein and water.[9, 10] When the proteins are removed and the nucleic acids tested, both are found to be equally sensitive to

UV, and nearly identical to the behavior of the sensitive strain. When the resistant strain is dried before irradiation, it becomes as sensitive to UV as the U2 strain, or the nucleic acids. Rewetting the virus restores its resistance. Siegel has taken advantage of these differential properties to investigate the behavior of virus and nucleic acid after they have infected host cells.

Leaves were inoculated with virus, or nucleic acid, and then irradiated at various times after inoculation. The results of Siegel's experiments are summarized by the data plotted in Figure 4. For convenience, the results of only a single, constant



KEY: ●, U1 nucleic acid; ⊡, U2 nucleic acid; ▽, intact U2; ⊙ and ×, intact U1.

FIG. 4.—Ultraviolet light survival of infective centers as a function of time after inoculation. Dose of UV: intact U2 and nucleic acid, 90 sec; U1, 5 min. From Siegel *et al.*, *Virology*, **3**, 554 (1957).

dose of UV are plotted. The behavior of the resistant strain is shown by the curve labeled U1. For approximately 5 hr after infection, the sensitivity of the virus remains unchanged from that exhibited by the virus outside the host. Then, with increasing time, the resistance to inactivation by UV continues to increase. The behavior of the sensitive strain, marked U2, is similar, although the ease by which its activity can be destroyed by lower doses of irradiation permits more detail in regard to its behavior to be obtained. The behavior of the sensitive strain remains the same as virus outside the host for about 2 hr, then sharply changes to a greater resistance. The change in resistance plateaus for about an hour, and then begins to increase again. The last increase in resistance is associated with a change in character of the inactivation curve, from a single hit, exponential curve, to a multiple target curve. This change in shape is interpreted to indicate that the virus

has commenced to multiply about 7 hr after inoculation. There is also reason to suspect that the entity which begins to multiply is different from the intact virus particle which initiated the infection, and it is presumed that the changes in sensitivity are associated with the release of nucleic acid from the protein subunits of the intact particle, followed by reproduction of the nucleic acid. Support for this view is found in Siegel's observation of infections commenced with nucleic acids. The curve marked NA depicts the behavior for both strains. The nucleic acids exhibit identical behavior which is in marked contrast to the inactivation behavior of the intact viruses from which the nucleic acids were obtained. The resistance to inactivation begins to increase almost immediately after inoculation, compared to the lag for each of the intact strains, and furthermore, Siegel was able to show that the inactivation curves for the nucleic acids both changed to a multi-target character in about 3 hours after infection, compared to 7 hours or more for intact virus.

Evidently, we can view the protein surrounding the nucleic acid of TMV as an impediment to the reproduction of the nucleic acid, the degree of impediment being a specific property of the TMV strain. We can imagine also that the different levels of resistance encountered with the intact strains are in some way associated with the release of nucleic acid from the protein subunits.

Thus, various lines of experimental evidence derived from many laboratories scattered throughout the world have converged to illuminate a remarkably detailed picture of the construction of an entity so small as to permit only its outlines to be visualized by the most exacting electron microscopy. Furthermore, some comprehension is being gained to relate this structure to its biological function.

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*BIOPHYSICAL STUDIES OF INFECTIOUS RIBONUCLEIC ACID FROM TOBACCO MOSAIC VIRUS**

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Following the brilliant discovery by Fraenkel-Conrat[1] and by Gierer and Schramm[2, 3] of the infectivity of the ribonucleic acid (RNA) of tobacco mosaic virus (TMV), considerable attention has been devoted to this material. However, a number of important questions persist. It is known that this material is unstable

both with respect to its physical properties[4] and to its infectivity. The origin of this instability is as yet obscure. The nature of the products of the instability has not been carefully investigated. Conflicting reports[5-8] have been issued with respect to the possibility that fragments smaller than the intact RNA of the virus can retain biological activity. The low specific infectivity of the viral RNA continues to leave open the question as to whether the infectivity may reside in a minor, special component of the preparation or whether the bulk of the preparation is, at least potentially, infective.[9]

Factors Influencing the Stability of the RNA.—The addition of 8 *M* urea[10] or of 10^{-3} *M* versene[11] to a fresh phenol preparation of RNA has no significant effect upon its infectivity. In addition, the presence of these agents was found to have no influence upon the rate of loss of infectivity at 25°C. However, the addition of certain metal ions[11] has been found to have significant effects upon the stability of the RNA at 25°C.

Of the cations studied (in concentrations of 5 ppm) the alkali metals (Na^+, K^+, Rb^+, Cs^+) and the alkaline earths (Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}) were ineffective.

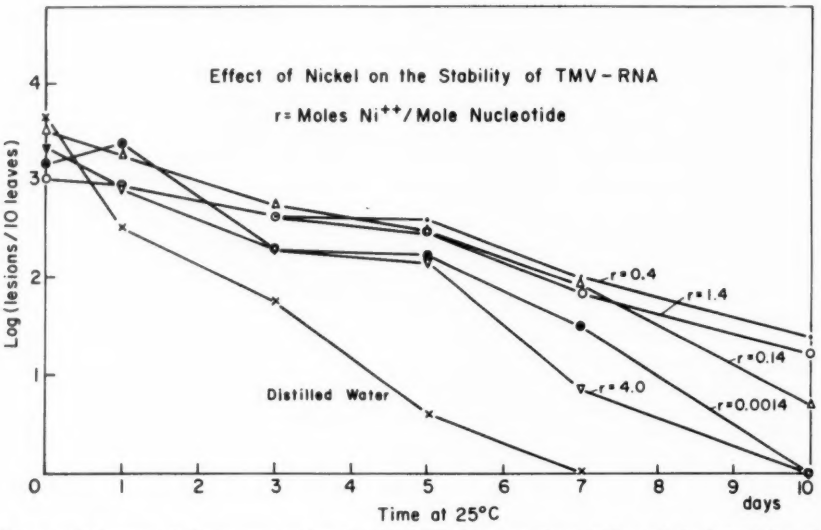


FIG. 1.—Influence of the concentration of added Ni^{++} ion on the stability of the infectivity of TMV RNA at 25°C.

Cupric and plumbous ions were toxic, destroying all infectivity. Chromous, ferrous, and manganous ions produced a slight stabilization of infectivity. Zinc, cobalt, and aluminum provided significant protection, while nickelous ion proved to be extraordinarily effective in stabilization of the infectivity (Fig. 1).

A broad optimum of protection was found about a concentration which provided one mole of nickel per mole of nucleotide. At this optimum concentration, the rate of loss of infectivity was reduced by a factor of four. However, a significant stabilization of infectivity was obtained at a concentration as low as 1 nickel ion

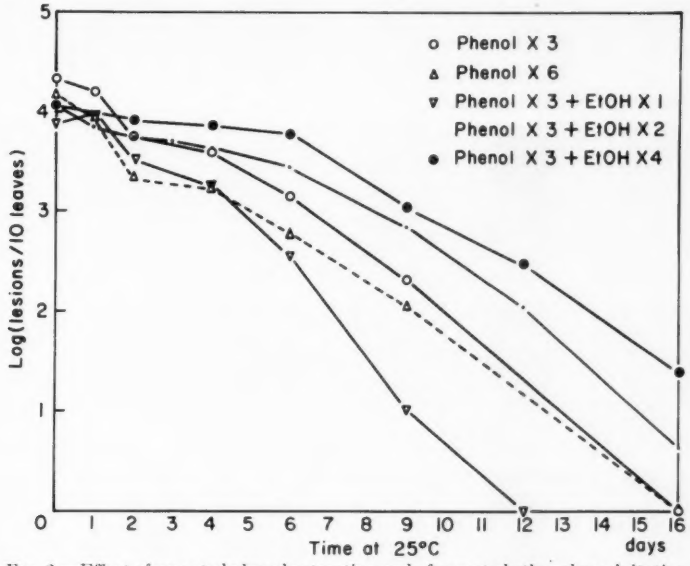


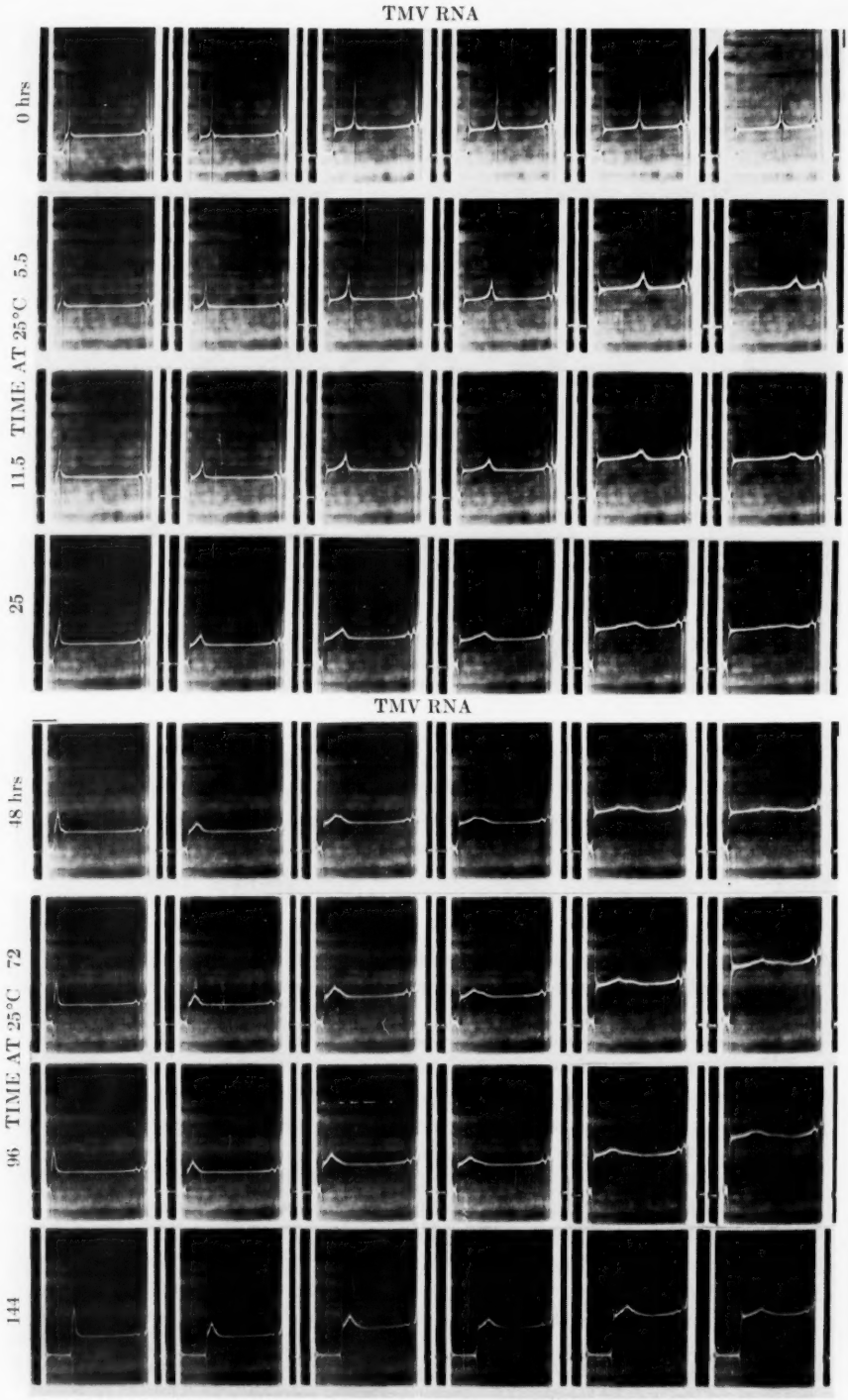
FIG. 2.—Effect of repeated phenol extraction and of repeated ethanol precipitation on the stability of the infectivity of TMV RNA at 25°C.

per 700 nucleotides or only 8 nickel ions per RNA molecule (this effect was produced with a Ni^{++} concentration of 5×10^{-9} g/ml).

The effectiveness of nickel at such low concentrations suggested that its action was to inhibit an extrinsic labilizing factor, rather than to stabilize the structure of the RNA molecule. Therefore, attempts were made to stabilize the infectivity by further purification of the RNA. As shown in Figure 2, it was found that while further phenol extraction was ineffective, repeated alcohol precipitation did result in a stabilization of the infectivity, presumably by removal or inactivation of a labilizing factor.

Ultracentrifugal Studies of the Degradation Products.—To learn more about the nature of the instability, a series of ultracentrifuge patterns was obtained as the biological infectivity disappeared upon standing at 25°C (Figs. 3 and 4). Accompanying the loss of infectivity, there is a physical disintegration of the RNA from its initial nearly monodisperse, rapidly sedimenting form, to a range of smaller sizes. During this degradation, a remarkably good correlation has been observed between the area remaining under the leading component (11S) at various times and the residual infectivity at those times (Fig. 5).

This correlation indicates rather conclusively that at no stage of the degradation do the smaller molecules carry any significant fraction of the infectivity. If the small fragments were active, the infectivity should fall off much more slowly than does the area of the leading component. However, this correlation does not prove the identity of the infective material with the material of the leading component, but merely indicates that the infective material and the RNA of the leading component are subject to disintegration, under these conditions, at very comparable rates.



FIGS. 3 and 4.—Ultracentrifuge patterns of a lx-ethanol-precipitated TMV RNA preparation after varving duration of storage in 0.02 *M* phosphate buffer, pH 7.5, at 25°C. The centrifuge patterns were obtained at 56,100 rpm, at 5°C, at a RNA concentration of 1 mg/ml.

Analysis of the centrifuge patterns obtained at various stages of degradation indicates that the breakdown of the RNA is not a completely random process, e.g., that the changes in the pattern do not appear to be those that would be expected from any process or enzyme acting completely at random along the length of the

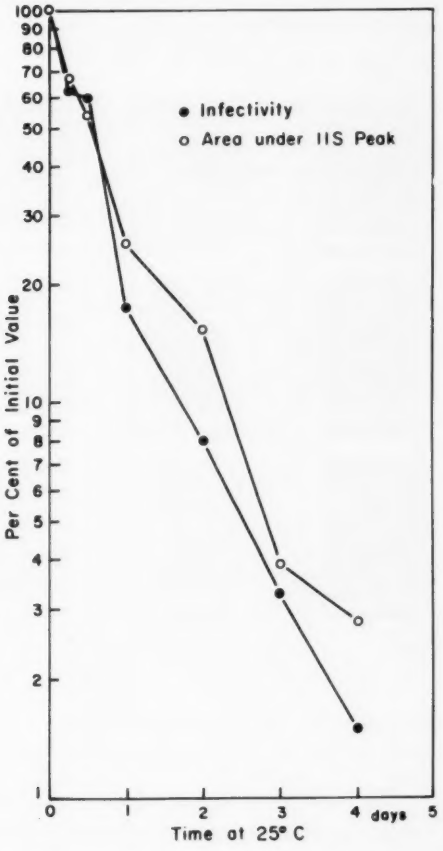


FIG. 5.—Correlation between the area of the leading component of the centrifuge pattern of TMV RNA remaining after varying periods at 25°C. with the infectivity remaining after similar periods.

RNA chain.[12, 13] The material initially present in the leading component appears, in early stages, in discrete, slightly slower components, and at later stages as more-or-less discrete, still slower components. This process is better observed in studies made upon the pattern of degradation of a 4-times alcohol precipitated preparation, in which the action of external labilizing factors has been minimized. Figure 6 illustrates the centrifuge pattern of such a preparation after 6 days at 25°C. Five distinct centrifugal components can be observed, of which the leading component corresponds to the residue of the original nearly monodisperse preparation. The existence of these discrete components provides clear evidence for the existence of subunits of the RNA, or more correctly, of preferred points of cleavage.

Fresco and Doty,[14] using synthetic polyribonucleotides, and Gierer,[12] using ribo-

nuclease digests of TMV RNA, have shown that at concentrations about 1 mg/ml the sedimentation constant for polyribonucleates varies as $M^{0.45}$. If, as a first approximation, the corrections for concentration dependence are omitted, the relative masses of the RNA components observed in the degradation pattern can be estimated from a log-log plot of the sedimentation rate versus the degree of polymerization; the slowest component is taken to be of unit degree of polymerization, and it is assumed that the observed sedimentation rates should lie on a line

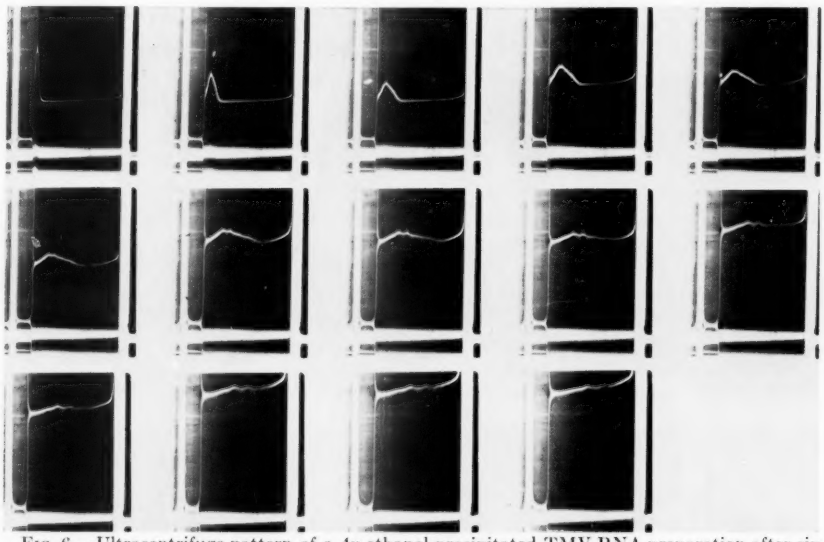


FIG. 6.—Ultracentrifuge pattern of a 4x-ethanol-precipitated TMV RNA preparation after six days at 25°C in 0.02 *M* phosphate buffer, pH 7.5. The centrifuge pattern was obtained at 56,100 rpm, at 5°C, at a RNA concentration of 1 mg/ml in a cell of 30 mm depth.

of slope 0.45. As illustrated in Figure 7, this approximate procedure results in a set of regular and integral degrees of polymerization for the observed components. This result implies, conversely, a regular pattern of breakdown of the RNA, at first into two-thirds and one-third pieces, then into one-sixth components and then again to smaller fragments, perhaps one-eighteenth. The validity of the approximation employed here, however, will have to be established by future work.[15]

Partition Cell Ultracentrifuge Studies.—A series of experiments has been conducted with the moving partition ultracentrifuge cell[22] in order to establish the identity,

TABLE 1

PARTITION CELL EXPERIMENTS WITH TMV RNA AT C = 1 mg/ml

Fresh Preparations

Manner of Preparation	UV Recovery, %	$Q_{int.}$	S(5°) Boundary	S(5°) UV	Infectivity
Phenol	91	0.41	12.6	11.2	12.0
Phenol	101	0.20	12.7	11.5	12.3
Phenol	100	0.37	12.2	11.1	16.5
Phenol	100	0.31	11.9	11.3	12.0
SDS	92	0.14	14.7	12.9	13.9
Av			12.8	11.6	13.3

or nonidentity, of the sedimentation rate of the bulk of the RNA preparation with the sedimentation rate of the infective element. In these experiments, three sedimentation constants can be determined: the S of the boundary or leading component by photography, the S for ultraviolet absorption by measurement of the proportion of the absorbing material remaining above the partition after a given centrifugal period, and the S for infectivity by measurement of the proportion of infectivity remaining above the partition after centrifugation.

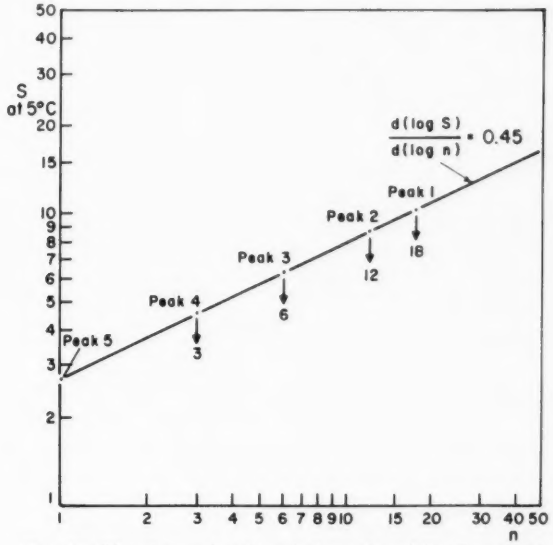


FIG. 7.—The observed sedimentation rates of the five centrifugal components of Figure 6 versus degree of polymerization, relative to the slowest component. It is assumed that the observed rates should lie upon a line of slope 0.45.

In Table 1 are summarized the results of five such experiments upon fresh RNA preparations, centrifuged at 1 mg/ml. The mean S for infectivity is 13.3 which is certainly not significantly different from the mean S of the schlieren component, 12.8. Table 2 summarizes the results of seven such experiments carried out, again at concentrations of 1 mg/ml, with aged preparations with varying degrees of

TABLE 2

PARTITION CELL EXPERIMENTS WITH TMV RNA AT C = 1 mg/ml

Aged Preparations

Manner of Preparation	Survival, %	UV Recovery, %	$Q_{inf.}$	S(5°) "Boundary"	S(5°) UV	S(5°) Infectivity
Phenol	10	...	0.06	11.7	9.6	14.5
Phenol	54	...	0.26	12.4	...	11.5
Phenol	61	110	0.04	12.2	8.1	16.7
SDS	59	100	0.26	...	7.2	12.1
SDS	43	97	0.17	12.0	8.4	12.4
SDS	4	113	0.05	11.5	7.5	15.5
SDS	40	94	0.05	12.9	6.8	15.6
Av				12.1	7.9	14.0

residual infectivity to as low as 4 per cent. In all cases the sedimentation rate for infectivity is essentially the same as is observed with the fresh preparations. Thus, the infectivity cannot be associated with any degradation products, but rather, in all cases, is associated with the residue of the original rapidly moving component.

However, these centrifugations were carried out using an RNA concentration of 1 mg/ml; at this concentration there is considerable molecular interaction and certainly not a free centrifugal movement. By the use of the more sensitive Xanthi strain of tobacco as test plants[23] it has been possible to carry out partition cell experiments at concentrations as low as 20 μg/ml, where the sedimentation rate of the RNA is the same as at infinite dilution. Under these conditions the sedimentation rate for the infectivity appears to exceed that of the ultraviolet boundary by a factor of 1.4 to 1.5 (Table 3).

TABLE 3

PARTITION CELL EXPERIMENTS WITH TMV RNA AT C $\leq$ 50 μgm/ml

Manner of Preparation	Survival, %	UV Recovery, %	Q_{int}	S(5°) Boundary	S(5°) UV	S(5°) Infectivity
Fresh Preparations						
Phenol	..	..	0.038	19.4	...	28
Phenol	...	90	0.072	...	18.2	31
Phenol	...	90	0.25	22.1	20.8	35.7
Aged Preparation						
Phenol	40	..	0.11	17.7	...	26

Interpreted literally, this result would indicate that the infectious element in these TMV RNA preparations comprises a very minor component with a sedimentation rate, at infinite dilution, 40–50 per cent greater than that of the bulk of the RNA. Since, however, these experiments amount to a fractionation of the RNA in which only the component of lower specific infectivity is recovered, it would be wise to withhold any conclusions until it shall have become possible also to recover a fraction of increased specific infectivity.

Summary.—TMV RNA preparations are subject to both extrinsic and intrinsic modes of degradation. The extrinsic mode can be reduced by traces of certain metals, notably nickel, and can be markedly minimized by purification of the RNA The intrinsic mode reveals a set of discrete and apparently uniformly spaced points of preferred cleavage of the RNA, which must reflect some interesting discontinuities of structure. No element smaller than the intact RNA has been found to be infective. Partition ultracentrifuge studies at low RNA concentrations cast doubt upon the identity of the bulk RNA of these preparations and the infective component.

Note added in proof: More recent experiments have demonstrated that the apparent large sedimentation coefficient for the infectious element in dilute solutions of RNA is an artifact, resulting from inactivation of the RNA when in dilute solution, both during and after the sedimentation. When this inactivation is taken into account, by appropriate controls, the sedimentation rate of the infectious element of the RNA in dilute solution is very nearly the same as the sedimentation rate of the ultraviolet absorption boundary.

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*TWO CONFIGURATIONS OF TOBACCO MOSAIC VIRUS–RIBONUCLEIC ACID**

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Infectivity is now generally accepted as an intrinsic property of viral ribonucleic acid (RNA) although the detailed physical and chemical requirements for this biological activity are still largely unknown. Recent evidence favors the concept that the infective RNA from tobacco mosaic virus (TMV) represents a single strand[1] of approximately 2 million molecular weight.[2] The question of the natural physical state of such an enormous macromolecule is of considerable interest. X-ray scattering data[3, 4] have adduced evidence that in TMV itself, the RNA is located at a radius of about 40 A° from the center of the cylindrical axis of the rod-shaped virus and is embedded within a matrix of protein subunits which are stacked in a helical array. As suggested by Ginoza,[5] it also seems likely that the RNA represents a helix of the same pitch within the virus. Since the polynucleotide retains biological activity even outside the protein, it is of considerable importance to ascertain if any specific molecular configuration must be retained for this activity. While none of the physical properties of infectious TMV-RNA in solution indicate that it possesses a characteristic secondary structure, evidence will be given suggesting that such a structure may be induced under the proper conditions.

Preparation of RNA in Free and Metal-Complexed Form.—The isolation of RNA

after detergent degradation of the virus followed by ammonium sulfate and alcohol precipitation has previously been described.[6] This product (preparation I) shows an absorbance (maximum, at 259 mμ) of about 25 for a 0.1 per cent solution, which is decreased by 20 per cent upon addition of magnesium or calcium salts (about 10^{-4} *M*), and to a slightly lesser extent (16 per cent) by 10^{-1} *M* monovalent cations. Such RNA also is surprisingly resistant to sedimentation in either water, or very low salt concentrations ($<10^{-4}$ *M*), as indicated by earlier studies in the preparative centrifuge[6, 7] and by the low sedimentation coefficient in the analytical centrifuge (<5 S). RNA preparations with identical properties are also obtained by the phenol procedure by substituting versenate (10^{-3} *M*) (ethylenediamine tetraacetate) for the phosphate buffer and/or redistilled phenol for the commercial phenol.

In contrast, the use of commercial phenol and phosphate buffer leads to an RNA preparation (RNA II) of about 20 per cent lower absorbance which is unaffected by the addition of mono or divalent cations.[8] Such preparations also sediment normally at low ionic strength.

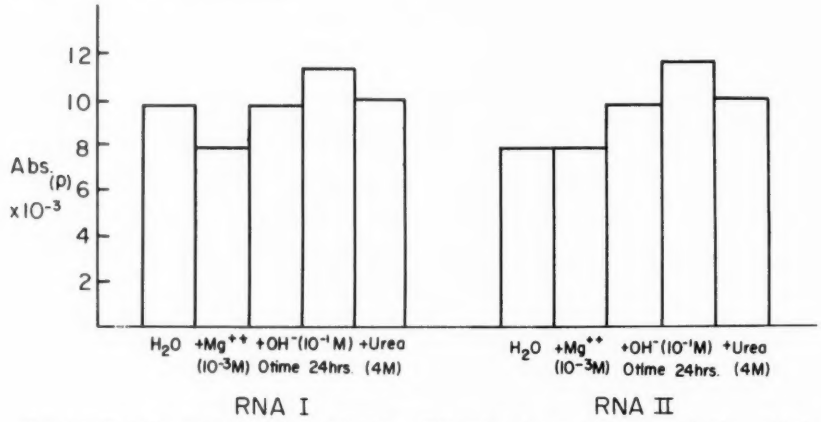

FIG. 1.—The $Abs._{(P)}$ of RNA II was based on RNA-phosphorus, after removal of bound ionic phosphate. The values are for the observed maxima which were at 258–259 mμ under all conditions.

Upon addition of urea, or alkali, the absorbance of RNA II rises immediately to the level of RNA I, which in contrast shows no effect upon such additions. Owing to hydrolysis, both preparations show a gradual 20 per cent increase in absorbance to a final $Abs._{(P)}$ value of about 12,000, under the influence of alkali[8] (see Fig. 1).

Spectrographic analyses of commercial and redistilled phenol (performed by American Spectrographic Laboratories, Inc., San Francisco) have provided an insight into the mechanism of formation of the two types of RNA by showing that the commercial phenol contained appreciable amounts of calcium and traces of other metals (Table 1). When redistilled phenol to which these metals had been added (as the chloride salts) was used in conjuncton with a phosphate buffer for the preparation of RNA, the resultant nucleic acid had properties identical with those of RNA II, as prepared with commercial phenol (Table 2). The addition of calcium chloride alone to redistilled phenol to give a concentration similar to that found

in commercial phenol (10^{-4} – 10^{-3} M) produced similar results. Comparison of the metal contents in the final RNA preparations showed that RNA II contained about 20 times as much calcium and up to 10 times as much of other metals as RNA I regardless of whether the former was prepared with commercial phenol or with redistilled phenol plus added metals (Table 3). Thus the RNA is definitely shown to exhibit a high affinity for metal ions, binding up to about one divalent

TABLE 1

ANALYSIS OF COMMERCIAL AND REDISTILLED PHENOL

Oxides of	Commercial (0.017 Per Cent Residue), *mg/10⁴ g Phenol*	Redistilled (0.0005 Per Cent Residue), *mg/10⁴ g Phenol*
Calcium	170	1
Iron	13	0.1
Sodium	10	0.63
Chromium	7	0.005
Nickel	4	0.035
Tin, aluminum	2	0.25
Magnesium	2	0.06
Other metals	0.3 or less	0.04 or less
Phosphorus	1020	25
Silicon	26	7.5
Boron	...	0.13

TABLE 2

PREPARATIONS OF TMV-RNA

Method of Preparation	Effect of Mg on OD (Maximum) Per Cent Change	Infectivity* Direct Per Cent	Infectivity* Reconstituted Per Cent
Commercial phenol, 0.02 M phosphate	−6 (−4)†, ‡	0.18	62
	−1 (0)‡	0.38	59
	−5‡	0.48	65
Redistilled phenol, 10^{-3} M versenate	−21†	0.09	72
	−19	0.40	42
	−21	0.22	52
	−19	0.17	52
Redistilled phenol, 0.02 M phosphate	−14	0.18	35
	−16	0.02	24
Redistilled phenol, 10^{-4} M $CaCl_2$, 0.02 M phosphate	−11	0.14	31
Redistilled phenol, 10^{-3} M $CaCl_2$, 0.02 M phosphate	0	0.10	43
	+2	0.51	57
Redistilled phenol, 10^{-3} M $MgCl_2$, 0.02 M phosphate	0	0.08	31
Redistilled phenol; Cr, Al, Fe, Ni (2×10^{-5} M each), 0.02 M phosphate	−14	0.28	58
Redistilled phenol; Ca (10^{-3} M); Cr, Al, Fe, Ni (2×10^{-5} M each); 0.02 M phosphate	+1	0.54	40
	0	0.12	14
	+1	0.13	98
	+1†	0.23	49

* Infectivity in reaction mixture in percentage of theoretical, based on RNA in sample and standard virus (averages of 12–48 half leaves).
† These samples were analyzed for metal content (see Table 3).
‡ Alcohol reprecipitated. Prior to this, there is usually little if any effect of added Mg on this type of preparation.

ion per 2 phosphorus atoms, in approximate agreement with the amount of metal required to cause maximal decrease in the absorbance of RNA I (Table 4).[8]

A few tests concerning the stability of linkage of the metal and the concomitant depression of absorbance showed partial reversal to occur during prolonged dialysis, or upon repeated alcohol precipitations. When versenate was present (0.01 M), RNA II was completely transformed to RNA I.

Dry weight and phosphorus analyses showed that RNA II retained some inorganic phosphate (about 20 per cent of its weight) in bound form, which was released

only concomitantly with the metal by treatment with versene, and from which it could then be separated by alcohol precipitation.

As Table 2 shows, there is no difference between the infectivity of RNA I and RNA II. If there seems little correlation between the two methods of determining the infectivity, this is partly due to the much greater variability of the results of the direct assay of RNA, as compared to those of virus or reconstituted virus, and the dependence of the RNA infectivity upon the physiological state of the host. Thus within a given test, the correlation between the infectivities of several RNA preparations and the reconstituted virus obtained from them is usually very much better than such a composite table of assays performed over several months would suggest.

TABLE 3

ANALYSIS OF TMV-RNA

Oxides of*	Preparation I (Random) (Redistilled Phenol Used in Preparation), %	Preparation II (Structured) (Commercial Phenol Used in Preparation), %	Preparation II (Structured) (Redistilled Phenol Plus Added Ca, Fe, Cr, Ni, Al), %
Calcium	0.25	5.6	4.5
Iron	0.01	0.09	0.01
Chromium	...	...	0.023
Aluminum	0.12	0.25	0.06
Nickel	...	...	0.045
Magnesium	0.07	0.75	0.6
Copper	0.001	0.004	0.007
Strontium	...	0.025	0.015
Barium	0.005	0.013	0.003
Tin	...	0.025	...
Manganese	...	0.025	0.03

* Potassium, sodium, phosphorus, silicon, boron not listed.

TABLE 4

EFFECT OF Mg^{++} AND Ca^{++} ON ABSORBANCE OF TMV-RNA I

Concentration of RNA		Ion Concentration Required for Depression of A by	
γ/ml	Absorbance (A)	About 10%	About 20% (Max)
128	3.2	0.4×10^{-4} *M* Mg	1×10^{-4} *M* Mg
12	0.31	0.4×10^{-5} *M* Mg	1×10^{-5} *M* Mg
32	0.80	1.2×10^{-5} *M* Mg	3×10^{-5} *M* Mg
32	0.80	1.2×10^{-5} *M* Ca	3×10^{-5} *M* Ca

Optical Rotation Studies.—The hypochromic effect exhibited by RNA I solutions upon addition of salts strongly suggests a change in molecular configuration, probably involving hydrogen bond formation. That RNA II, the metal complexed preparation, does not exhibit this phenomenon may be explained by the presence of extensive hydrogen bonding in the molecule as it is prepared. If the difference between the two types of RNA is actually due to hydrogen bonding, then it would be of interest to know if the hydrogen bonds form in a random fashion or if they give rise to an ordered structure such as, for example, the helical model proposed by Ginoza.[5]

The optical rotatory dispersion of the two types of RNA was studied in various ionic media over the wave-length region of 320 to 600 mμ. RNA II exhibits a high positive rotation in water solution, with the specific rotation at 540 mμ ($[\alpha]_{540}$) equal to 224°. No change in optical rotation was observed upon the addition of either sodium chloride or magnesium chloride to the solution. The dispersion fol-

lows a simple one term Drude equation with the critical wave length (λ_c) equal to 240 mμ.

Dispersion curves obtained for different preparations of RNA I exhibited considerable variation; this apparently resulted from slight differences in the conditions of preparation or from the presence of contaminants in the water used in making the solutions. One sample of high molecular weight infectious RNA I, for example, had $[\alpha]_{540} = 20°$ when diluted with doubly distilled water. A more typical value for $[\alpha]_{540}$ is between 70° and 80°. Again the dispersion curve fits a single term Drude equation and λ_c in this case is equal to 283 mμ. Addition of sodium chloride to the RNA I solution increases the rotation gradually to a maximum of $[\alpha]_{540} = 180°$ at about 0.1 *M* sodium chloride. Further addition of sodium chloride causes no change in the rotation. Magnesium chloride produces a similar effect on the rotation with the maximum of $[\alpha]_{540} = 224°$ occurring at 0.002 *M* to 0.004 *M* magnesium chloride. Under these conditions, the rotatory dispersion of RNA I is identical with that obtained for RNA II.

The rotatory dispersion of RNA II was also investigated as a function of temperature. A definite transition was found to occur at around 60°C as evidenced by a sharp drop in the optical rotation. Studies on model compounds[9] indicate that no depolarization or refraction of the light beam occurs even up to temperatures of 90°C. Since the observed effect far exceeded that which could be explained on the basis of a change in the refractive index of the medium, it must be inferred that a change in molecular configuration had occurred. The complete reversibility of the effect eliminated heat degradation as a possible cause; therefore one might attribute it to a breakdown of some secondary structure.

These results are consistent with the hypothesis that RNA I exists in water as an extended random coil, RNA II as a helical structure, and that the two may be interconverted by appropriate choice of solvent. The greater efficiency of magnesium chloride as compared to sodium chloride in causing an increase in the rotation and a decrease in the absorption at 258 mμ suggests that divalent ions act as chelating agents, holding the nucleic acid molecule in a configuration which is favorable for intramolecular hydrogen bond formation; monovalent ions probably serve only to damp out charge repulsions, thereby allowing hydrogen bonds to form by virtue of the proximity of the hydrogen bonding bases.

Ultracentrifugation Studies.—Sedimentation studies were performed in a Spinco Model E analytical ultracentrifuge at a speed of 59,780 rpm. A Spinco Model R Analytrol was used to translate ultraviolet absorption photographs to a tracing of concentration versus distances in the cell. A 0.01 *M* phosphate buffer at pH 7.0 was chosen as the solvent, this concentration being sufficient to damp out some of the intermolecular charge effects, without causing a substantial conversion of RNA I to RNA II.

Ultracentrifugal analysis of several samples of RNA II, the presumably structured configuration, indicated that from 40 to 60 per cent of the nucleic acid was present as an apparently homogeneous component with a sedimentation coefficient of 22 S, the amount depending on the homogeneity of the TMV used in the preparation; the remainder consisted of heterogeneous material with a sedimentation coefficient distribution between 15 S and 22 S. In contrast, RNA I, the random coil preparation, showed greatly varying amounts of this homogeneous component,

the patterns frequently presenting only a continuous distribution of material sedimenting between 5 S and 20 S. In the light of results reported below, this variation may be accounted for by the presence of varying trace quantities of divalent ions in the solution.

Despite the great difference in sedimentation behavior, RNA I samples containing no 22 S component exhibited, as stated, similar infectivities as RNA II preparations (Table 2). When two RNA I samples were reacted with TMV protein, reconstituted virus was obtained to the extent that about 30 and 50 per cent of the original nucleic acid had been incorporated into homogeneous TMV rods, as determined by ultracentrifugation of the reaction mixture. The high infectivity of such reconstituted virus (Table 2) also supported the belief that a high proportion of the free RNA I in solution, notwithstanding its sedimentation pattern, was homogeneous with respect to molecular weight.

The sedimentation behavior of random RNA was therefore studied in 0.01 M phosphate to which various concentrations of magnesium chloride had been added. In a typical sample having no 22 S component in 0.01 M phosphate alone, the presence of this component was first detectable in 10^{-5} M magnesium chloride (RNA concentration = 25 γ/ml). In 5×10^{-5} M magnesium chloride about 15 per cent of the RNA appeared as a 22 S component. When the magnesium chloride concentration was increased to 10^{-4} M, the sedimentation pattern of the sample showed 50 per cent of the 22 S component and was similar to the pattern given by structured RNA, except for the presence of some aggregated material. Further increases in magnesium chloride concentration simply caused more aggregation of the nucleic acid. The addition of magnesium chloride to other RNA I preparations produced similar qualitative effects, although quantitative differentiation between heterogeneity of molecular weight and that of shape has not been possible.

These results complement the optical rotation and absorption studies and demonstrate the presence of a secondary structure for RNA in solution, the extent of which is dependent upon the environment. They unfortunately also suggest that the sedimentation behavior in 0.01 M buffers of an extremely long unpaired polynucleotide chain is difficult to predict and interpret.

Summary.—Under the influence of very low concentrations of divalent cations, or higher concentrations of monovalent ions, TMV-RNA was shown to undergo changes in optical density at 258 mμ, in optical rotation, and in sedimentation behavior. These changes suggested the transformation from a random coil to a more orderly configuration.

The use in the isolation of the RNA of phenol containing traces of metals, mainly calcium ions, led directly to the structured type of preparation, which was found to contain appreciably more metal than was found in the material isolated from pure phenol. Both types of preparations were similarly infectious, and each could be transformed into the other reversibly.

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*THE CHEMICAL NATURE OF THE RNA-AMINO ACID COMPOUND FORMED BY AMINO ACID-ACTIVATING ENZYMES**

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Specific enzymes which catalyze the formation of amino acyl adenylates have been isolated from bacterial, plant, and animal sources.[1–5] More recently it was found that these enzymes also catalyze the formation of an amino acyl-RNA compound, and the evidence from a number of laboratories[6–10] favors the mechanism shown in equations (1) to (3).

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{amino acyl-AMP} + \text{PP} \tag{1}$$

$$\text{Amino acyl-AMP} + \text{RNA} \rightleftharpoons \text{amino acyl-RNA} + \text{AMP} \tag{2}$$

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{amino acyl-RNA} + \text{AMP} + \text{PP}. \tag{3}$$

The acceptor RNA, which represents about 10 per cent of the cellular RNA of *Escherichia coli*, is of relatively low molecular weight.[11] Each amino acid is linked to a specific site on the RNA and these acceptor sites function independently of one another.[6–8]

Recently, Hecht *et al.*[12] showed that the acceptor activity of liver RNA required a specific terminal sequence of nucleotides, i.e., adenylyl 5′-3′-cytidylyl-5′-3′-cytidylyl-5′-3′-RNA (RNApCpCpA[13]). It seemed likely that this grouping provides either the point of attachment of amino acids to the RNA or that this terminal sequence is required for the binding of the RNA to the activating enzyme. The experiments described in the present report were designed to distinguish between these two possibilities.

Our results show that the leucine-, valine-, and methionine-activating enzymes link the respective amino acids to the RNA through the 2′- or 3′-hydroxyl group of the ribose of the terminal nucleotide bearing the unesterified 3′-hydroxyl group. We have identified the terminal nucleotide of the chain which accepts leucine as adenylic acid by isolating leucyl-2′- or 3′-adenosine from RNase digests of leucyl-RNA. From the behavior of the bound amino acids to treatment with hydroxylamine,[6, 7, 14] we have inferred that the linkage is an acyl ester. Based on the present

concept of the structure of RNA, i.e., that there is no branching of the polynucleotide chain, we have concluded that there is only one amino acid bound per polynucleotide chain. These findings, together with the observations of Zachau *et al.*[14] that a terminal adenylic acid unit is also the acceptor for leucine in liver "soluble" RNA, support the idea that the adenylic acid in the terminal sequence adenylyl 5′-3′-cytidylyl 5′-3′-cytidylyl 5′-3′-RNA may be the acceptor for all amino acids[12] and that the remainder of the RNA chain functions in determining which amino acid is linked to which chain.

General Methods and Materials.—Amino acid-activating enzymes specific for L-valine, L-methionine, and L-leucine were each purified on the basis of the ATP-PP^{32} exchange reaction,[2] from extracts of *E. coli* by methods to be reported later. With the purified enzymes, the rate of ATP-PP^{32} exchange with any other amino acid was less than 5 per cent of that observed with the specific amino acid.[15] Snake venom phosphodiesterase, free of phosphomonoesterase activity, was prepared by the method of Koerner and Sinsheimer;[16] semenphosphomonoesterase, free of phosphodiesterase activity, was obtained from Dr. L. A. Heppel of the National Institutes of Health; and crystalline pancreatic RNase was purchased from Worthington Biochemicals Corp. The amino acid-acceptor RNA was isolated from dried cells of *E. coli* by a procedure to be published later.

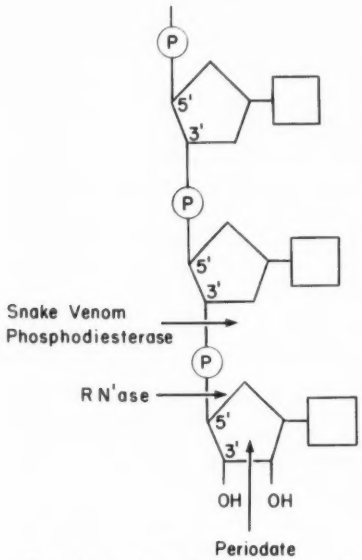


FIG. 1.—Action of periodate, snake venom phosphodiesterase, and RNase on a portion of a typical polynucleotide chain.

The quantity of amino acid linked to RNA was determined by incubating the appropriate C^{14}-labeled amino acid with an excess of the specific amino acid-activating enzyme, ATP, Mg^{++}, and RNA, and measuring the amount of radioactivity precipitated by 0.35 *N* perchloric acid, or by an ethanol-salt mixture (67 per cent ethanol–0.5 *M* NaCl). Under these conditions the amount of amino acid precipitated was directly proportional to the amount of RNA added[7] and we have used this value as a measure of the total number of specific binding sites for a given amino acid present in the RNA. On several occasions a mixture of C^{14}-labeled amino acids together with an unfractionated extract of *E. coli* was used to assay the RNA for total amino acid-acceptor activity.[7] Further details are presented with the individual experiments. Inorganic phosphate was measured by the method of Fiske and Subbarow.[17]

Experimental Results.—Requirement for specific terminal nucleotide unit for linking amino acids to RNA: Removal from the polynucleotide chain of the terminal unit bearing the unesterified 3′-hydroxyl group can be accomplished by oxidative cleavage of the ribose moiety with periodate (see Fig. 1), followed by alkaline

decomposition of the oxidized unit and enzymatic removal of the resulting phosphomonoester groups.[18] This procedure not only removes the original terminal nucleotide, but regenerates a new end having a free 3′-hydroxyl group. Treatment of the isolated acceptor-RNA from *E. coli* in this manner results in essentially complete loss of acceptor activity (Table 1). Periodate treatment alone (line 2) is also sufficient to inactivate the RNA, showing that the RNA must have an intact as well as a specific terminal nucleotide unit in order to accept amino acids.

Snake venom phosphodiesterase can degrade deoxy- and ribo-oligonucleotides, one nucleotide at a time, starting from the end bearing the free 3′-hydroxyl groups on the pentose.[19–22] Moreover, the enzyme attacks available ends at random rather than degrading one complete chain at a time.[21, 22] Phosphodiesterase digestion of amino acid-acceptor RNA results in essentially complete loss of acceptor activity when approximately 5 per cent of the nucleotides have been split off (Fig. 2). By contrast, spleen phosphodiesterase which degrades oligodeoxynucleotides from the nucleotide end bearing the free 5′-hydroxyl group,[21] but whose action on polyribonucleotides is not yet established, produced only 20 per cent loss of acceptor activity for an equivalent amount of degradation.

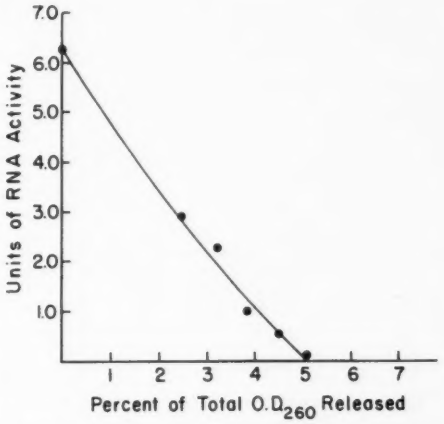


FIG. 2.—Degradation of the amino acid-acceptor RNA by snake venom phosphodiesterase.

Snake venom diesterase digestion: The final volume of 0.14 ml contained 0.14 *M* tris buffer, pH 8.7, 0.007 *M* $MgCl_2$, 0.5 mg of RNA, and 6.3 units of snake venom phosphodiesterase. The mixture was incubated at 37° and samples were removed at 0, 5, 10, 20, 30, and 60 min and heated for 2 min at 100°. The amount of degradation was determined by measuring the optical density at 260 mμ of the supernatant fluid following the addition of perchloric acid to the heated aliquot. Another portion of each sample was assayed for its ability to accept amino acids, using the mixed C^{14}-amino acids and unfractionated *E. coli* extract as previously described. The ordinate is expressed as units of RNA activity and 1 unit corresponds to the incorporation of 10^3 cpm under the standard conditions. Incubation of the RNA in the absence of the phosphodiesterase led to no loss in activity.

These two experiments show that a specific nucleotide unit at the end of the RNA chain having a free 3′-hydroxyl group is essential for the activity of the acceptor-RNA fraction from *E. coli*.

The terminal nucleotide as site of attachment for amino acids: If either of the hydroxyl groups of the ribose on the terminal nucleotide is the site of attachment for an amino acid, then treatment of the amino acid-RNA compound with periodate should not result in loss of acceptor activity. Specifically, this hypothesis states that if the leucyl-RNA is exposed to periodate, all sites other than the one specific for leucine should be inactivated and the leucine site should be protected. Table 2 describes an experiment in which leucyl-, valyl-, and methionyl-RNA were each treated with periodate, recovered by precipitation, and freed of the bound amino acids. The RNA was then tested for the ability to accept each of these amino

TABLE 1

EFFECT OF PERIODATE OXIDATION ON AMINO ACID-ACCEPTOR ACTIVITY OF *E. coli* RNA*

RNA Sample	Amount of Amino Acid Linked to RNA, mμ mole per μmole of Nucleotide	
	Leucine	Valine
1. Original RNA	0.67	0.39
2. Periodate-treated RNA	0.00	0.02
3. Two incubated at pH 10	..	0.02
4. Three treated with phosphomonoesterase	0.00	0.02
5. Original RNA incubated at pH 10	..	0.33
6. Original RNA treated with phosphomonoesterase	0.69	0.37

The periodate treatment of the RNA was carried out as follows: 30 mg of RNA in 0.1 *M* succinate buffer, pH 5.6, was incubated for 30 min at 20° with 2 to 5 μmoles of sodium metaperiodate. This represents a 2- to 5-fold excess of periodate over the calculated number of end groups assuming that there are on the average 100 nucleotides per RNA chain.[11] The RNA was recovered by precipitation with alcohol, dialyzed against water, and then used as such, or the solution was made 0.1 *M* with glycine buffer, pH 10.3, and incubated at 20° for 16 hr. The alkali-treated RNA was recovered by precipitation with the ethanol-salt mixture. Phosphatase treatment was carried out on the alkali-treated RNA as follows: approximately 20 mg of the RNA was incubated at 37° with 22 units of purified semen phosphomonoesterase (1 unit = 1 μmole of phosphate removed from adenosine 3′-phosphate per hour) at pH 5.0 in the presence of 0.01 *M* Mg^{++}. Estimation of the amount of inorganic phosphate liberated showed that 0.61 μmole of phosphate was split off. This corresponded to 94 per cent of the amount expected on the basis of the assumed chain length of 100 nucleotides. The polynucleotide minus the original terminal nucleotide was reisolated by precipitation with ethanol and salt and assayed as described below.

The amount of amino acid linked to RNA was determined as follows: The reaction mixture volume was 0.5 ml and contained 0.1 *M* sodium cacodylate buffer, pH 7.0, 0.002 *M* $MgCl_2$, 0.01 *M* ATP, 0.2 mg of bovine serum albumin, 0.002 *M* glutathione, 0.0003 *M* C^{14}-DL-leucine or C^{14}-DL-valine (specific activity 3.5 to 6.0 × 10^6 cpm per μmole) and either 0.9 μg of the purified leucine-activating enzyme or 0.5 μg of the valine-activating enzyme. Incubation was for 20 min at 30°, at which time the incorporation was complete. The RNA was precipitated with ethanol-salt, washed, and counted in a windowless-gas-flow counter with appropriate self-absorption corrections. Testing of control mixtures of inactivated acceptor-RNA and active RNA under these conditions showed no decrease in the yield of RNA-amino acid formed.

* This loss of amino acid-acceptor activity occurs for all amino acids since the incorporation of a mixture of C^{14}-labeled amino acids in the presence of a crude extract of *E. coli* containing many activating enzymes[5] is likewise prevented by treatment with periodate.

TABLE 2

ASSAY OF RNA ISOLATED AFTER TREATMENT OF AMINO ACID-RNA COMPOUNDS WITH PERIODATE

Amino Acid Tested	Original RNA		Leucyl-RNA		Valyl-RNA		Methionyl-RNA	
	Periodate Treatment							
	+	−	+	−	+	−	+	−
	Amino Acid Incorporation, mμmole per μmole of Nucleotide							
Leucine	0.01	0.59	0.56	0.54	<0.01	0.54	<0.01	..
Valine	0.04	0.30	0.04	0.28	0.23	0.30	0.03	..
Methionine	<0.005	0.13	<0.005	0.12	<0.005	0.13	0.15	0.15

The different C^{14}-amino acid-RNA compounds were prepared by scaling up the usual assay reaction mixtures described in Table 1. Five to 10 mg of the amino acid-RNA compound were incubated with periodate as described previously and then reisolated by alcohol precipitation and dialysis. Approximately 85 to 90 per cent of the optical density units and radioactivity was recovered. The C^{14}-amino acids were removed by treatment with 0.1 *M* glycine buffer, pH 10.3 at 30° for 60 min and the amino acid-free RNA was recovered by precipitation. The yield of RNA was between 80 and 90 per cent of the initial input. The different RNA preparations were then assayed for their ability to accept leucine, valine, and methionine by the methods described previously. In the case of the methionine assay, 0.00025 *M* C^{14}-L-methionine (specific activity 4.9 × 10^6 cpm per μmole) and 30 μg of a purified L-methionine activating-enzyme from *E. coli* were used.

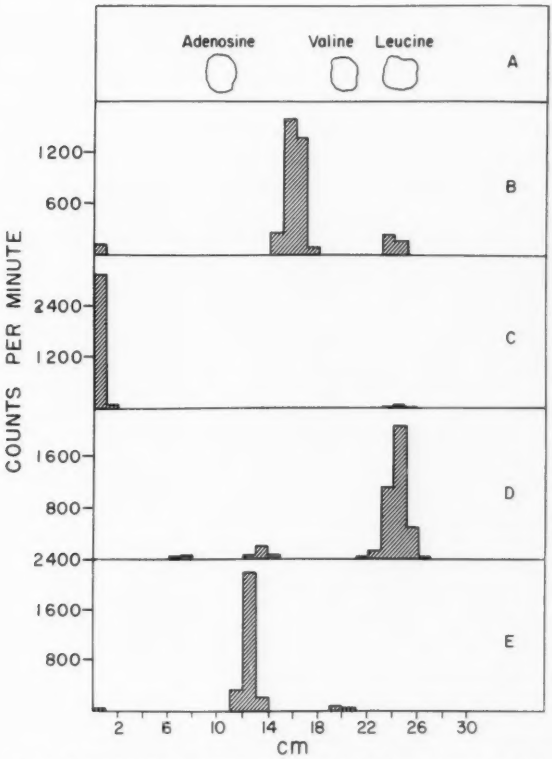


FIG. 3.—Analysis of chromatograms of RNase digests of C^{14}-leucyl and C^{14}-valyl RNA.

(*A*) Adenosine, valine, and leucine markers. (*B*) C^{14}-leucyl-RNA + RNase. (*C*) C^{14}-leucyl-RNA. (*D*) C^{14}-leucyl-RNA treated with 0.01 *N* KOH. (*E*) C^{14}-valyl-RNA + RNase.

The samples used for chromatography were prepared as follows: Approximately 0.5 mg of C^{14}-leucyl- or valyl-RNA, prepared as described in Table 1, were incubated with 0.3 μg of crystalline RNase at pH 5.8, for 15 min at 30°, and then an aliquot was placed on Whatman No. 1 filter paper strips and chromatographed at 3° with sec-butanol:formic acid:H_2O (70:10:20).[25] Another sample of leucyl-RNA was incubated in 0.01 *N* KOH for 5 min at 20°. This procedure was insufficient to cause any breakdown of the RNA. Following chromatography, the individual strips were cut into 1-cm sections, eluted with 0.05 *N* NH_4OH, and the eluates counted. The amount of radioactivity per 1-cm strip is plotted against the distance along the paper.

Not shown here are chromatograms of C^{14}-valyl-RNA, and C^{14}-valyl-RNA treated with alkali. Valyl-RNA remained at the starting point, while in the latter experiment all the radioactivity migrated the same distance as free valine.

acids. The results show that these amino acids protect only their own sites on the RNA against inactivation by periodate. Based on current ideas of polynucleotide structure, the only periodate-sensitive groups are the terminal nucleotides bearing unesterified 3′-hydroxyl groups. Assuming that the polynucleotide chain is un-

branched, we can conclude that such a chain has a single amino acid-binding site and that this is the 2′- or 3′-hydroxyl group of the terminal nucleotide having the free 3′-hydroxyl group. Moreover, since there are specific sites for each different amino acid, there must be a different polynucleotide chain for each amino acid.

Identification of the terminal nucleotide to which leucine is linked: Pancreatic ribonuclease (RNase) digestion of RNA yields nucleosides from those end groups which have a free 3′-hydroxyl group and which are adjacent to a pyrimidine nucleo-

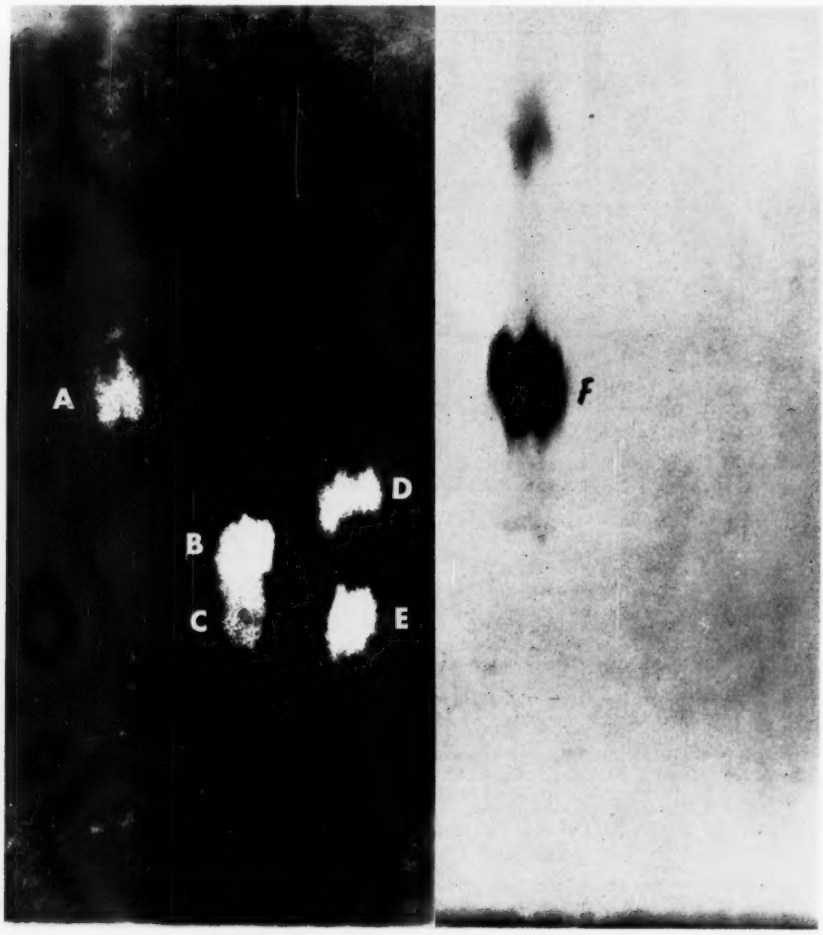
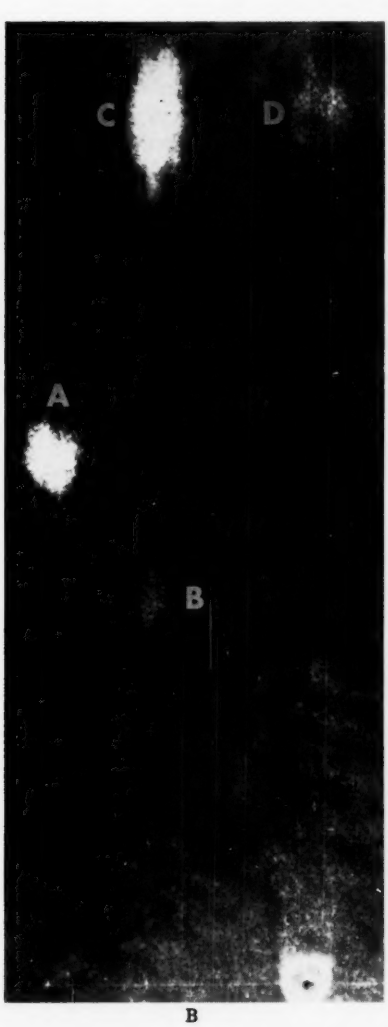


FIG. 4.—Chromatographic identification of leucyl-adenosine as product produced by RNase digestion of leucyl-RNA.

(*a*) Chromatogram and radioautogram of the isolated C^{14}-leucyl-adenosine and ribonucleoside markers in the solvent described in Figure 3. The various spots are (*A*) C^{14}-leucyl-adenosine, (*B*) adenosine, (*C*) cytidine, (*D*) uridine, and (*E*) guanosine. At the right (*F*) is the X-ray overlay showing the position of the radioactivity. The small amount of radioactivity at the top of the radioautogram represents free leucine derived from breakdown of the leucyl-adenosine during the chromatographic run.

tide.[24] It was reported earlier[7] that amino acids linked to RNA were converted from an acid-insoluble form to an acid-soluble form. Chromatography of the acid-soluble fraction from RNase digests of C^{14}-leucyl- and C^{14}-valyl-RNA showed that all of the amino acids migrated to a region slightly ahead of that occupied by the free nucleosides and behind the free amino acids (Figs. 3, *A*, 3, *B*, and 4, *A*). The



B

C

(*b*) Chromatogram of the alkaline hydrolysate of the C^{14}-leucyl-adenosine in *n*-butanol:isobutyric acid:NH_4OH.[26] The leucyl-adenosine was exposed to 0.01 *N* KOH for 5 min at 30°. The various spots are: (*A*) uridine, (*B*) guanosine, (*C*) adenosine, and (*D*) hydrolysate.

(*c*) Chromatogram of the acid-hydrolysate of the isolated adenosine in *n*-butanol:isobutyric acid:NH_4OH.[26] The adenosine was hydrolyzed in 1 *N* HCl for 15 min at 100°. The various spots are: (*A*) adenosine, (*B*) guanosine, (*C*) uracil, (*D*) adenine, and (*E*) hydrolysate.

leucine-bound component was isolated by successive paper chromatographic runs (Fig. 4, *A*) and subjected to dilute alkaline hydrolysis. All of the radioactivity now migrated with free leucine and the ultraviolet-absorbing material chromatographed as adenosine (Fig. 4, *B*). The isolated adenosine, when hydrolyzed with acid, gave quantitative recovery of an ultraviolet-absorbing material which was identified as adenine (Fig. 4, *C*). We infer that the leucine was linked to one of the two *cis*-hydroxyl groups of the ribose since the R_f of the leucyl-adenosine was unaffected by treatment with periodate while free adenosine, subjected to the same procedure, gave a spot with an altered R_f (0.37 as compared to 0.30 in the butanol-formic acid solvent). We have concluded from this experiment that the terminal nucleotide which binds leucine is, as was found by Zachau *et al.*[14] with liver "soluble" RNA, adenylic acid, and, moreover, from the known reactivity of the bound amino acids to hydroxylamine[6, 7, 14] that the linkage is from the amino acid carboxyl group to the 2′- or 3′-hydroxyl group of the ribose.

Discussion.—Amino acid-activating enzymes catalyze a two-step reaction by which amino acids become linked to specific sites of RNA. The present results, together with those of Hecht *et al.*[12] and Zachau *et al.*[13] show that these sites are terminal nucleotides bearing a free 3′-hydroxyl group on the ribose, and that the attachment of the amino acid to the RNA is very likely by an ester linkage. If, as suggested by Hecht *et al.*,[12] all amino acid-acceptor ends are terminated by an identical sequence of at least three nucleotides (RNApCpCpA), then either the structure or the composition and sequence of nucleotides in the remaining portion of the chain must determine which amino acid is linked to which chain. It seems likely that this specificity results from the interaction of a portion of the polynucleotide chain with specific amino acid-activating enzymes. Consistent with such a hypothesis are preliminary studies which show that the polynucleotide, from which the terminal nucleotide has been removed by the successive action of periodate, alkali, and phosphomonoesterase, competitively inhibits the rate of amino acid incorporation into active RNA, but does not affect the yield. The periodate oxidation technique we have used has enabled us to make preparations which are specific for single amino acids but which still contain the inactive chains. It is clear that the answer to the specificity problem will require the isolation of homogeneous preparations of polynucleotide chains specific for a single amino acid.

It may be of some interest to point out one difference between the properties of the isolated mammalian and the bacterial amino acid-acceptor RNA. Zachau *et al.*[14] and Hoagland[27] have reported that the isolated "soluble" RNA from liver is already saturated with respect to many amino acids and the incorporation of labeled amino acids represents an exchange process. This is not the case with the acceptor-RNA as we have isolated it from *E. coli*, for if amino acids were already linked to the RNA it would not have been possible to completely inactivate the original RNA with periodate. This difference between the mammalian and bacterial RNA preparations may be simply the result of the different procedures used to isolate the RNA rather than some fundamental difference in the two types of RNA.

Zachau *et al.*[13] have pointed out that this type of acyl ester linkage, i.e., the amino acid-ribonucleotide compound, represents a new type of "high-energy bond." Studies on the measurement of the equilibrium constant for the enzymatic reaction

linking valine to RNA indicate a value of 0.32 at pH 7.0 at 30°,[28] showing that only a small free energy change is involved in forming an amino acyl-RNA compound from ATP, the free amino acid, and RNA.

Summary.—Purified leucine-, valine-, and methionine-activating enzymes from *E. coli* catalyze the transfer of these amino acids to a specific fraction of RNA according to the following general equation:

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{amino acid-RNA} + \text{PP} + \text{AMP}.$$

The acceptor-activity of the RNA is lost after destruction or removal of the terminal nucleotide having the free 3′-hydroxyl group by periodate or the action of snake venom phosphodiesterase. If an amino acid is linked to the RNA prior to treatment with periodate, the acceptor site specific for the bound amino acid is protected against inactivation while all others are destroyed. Treatment of leucyl-RNA with pancreatic RNase results in the liberation of leucyl 2′- or 3′-adenosine. All of these observations are consistent with the hypothesis that each amino acid is linked to a specific polynucleotide chain through linkage of the amino acid to the 2′- or 3′-hydroxyl group of the terminal nucleotide of the chain.

* This work was supported by a grant from the United States Public Health Service. The following abbreviations have been used: ATP, adenosine triphosphate; AMP, adenosine 5′-monophosphate; PP, inorganic pyrophosphate; RNA, ribonucleic acid.

† American Cancer Society Postdoctoral Research Fellow.

‡ National Science Foundation Predoctoral Research Fellow.

§ U.S. Public Health Postdoctoral Research Fellow.

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*A PROCEDURE FOR DETECTING CARRIERS OF GALACTOSEMIA**

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Introduction.—Galactose-1-phosphate-uridyl transferase (transferase) catalyzes a reversible hexose exchange which results in the formation of uridine diphosphate galactose (UDPGal) and glucose-1-phosphate (Glu-1-P) from galactose-1-phosphate (Gal-1-P) and uridine diphosphate glucose (UDPGlu):

$$\text{Gal-1-P} + \text{UDPGlu} \rightleftharpoons \text{UDPGal} + \text{Glu-1-P}.$$

This enzyme is absent or deficient in the erythrocytes of galactosemics,[1] thus partially blocking the normal metabolism of galactose. As much of the ingested galactose is not excreted by galactosemics,[2,3] the possibility of an incomplete metabolic block or of an alternative pathway of galactose metabolism must be considered. Such an alternative pathway has been demonstrated in liver.[4]

Methods have been devised to detect galactosemics, based upon measuring (1) galactose tolerance, (2) the Gal-1-P which accumulates in the red cells,[5] or (3) the transferase level in the red cells.[1] Standard galactose tolerance tests have revealed that one of the parents of galactosemic children usually show a lower tolerance to galactose.[6] It might be expected therefore, that the transferase level of red cells of these parents should also be lower. Nevertheless, Anderson *et al.*[7] were unable to detect any difference (in the enzyme level of the red blood cells) between parents of galactosemic children and normal individuals. Presuming that this result may be due to limitations of the enzyme assay employed, the quantitative aspects of the procedure have been investigated.[8] Some important modifications have resulted in a more satisfactory procedure, which, when applied to a study of known galactosemics and their families, reveals that heterozygotes indeed have a transferase level intermediate between the affected and the normal individuals. Hsia *et al.*[9] also have recently presented evidence that transferase levels tend to be lower in the parents of galactosemics as compared to normal individuals.

The description and application of the modified transferase assay are the subject of this communication.

Methods.—The initial assay for transferase was based on the original procedure of Kalckar *et al.*[1] and consisted of incubating hemolyzates of red cells with Gal-1-P

and UDPGlu. The residual UDPGlu was determined spectrophotometrically by relating the quantitative reduction of diphosphopyridine nucleotide (DPN) to UDPGlu in the dehydrogenase catalyzed oxidation of UDPGlu to UDPGlucuronic acid. The UDPGal formed was determined by the addition of a purified UDPGal-4-epimerase to the same reaction mixture after the dehydrogenase catalyzed reaction had ceased. This assay resulted in the qualitative detection of the transferase, but was not suitable for further studies requiring more quantitative enzyme levels.

The modified assay, as now employed, is similar to the most recent enzymatic assays published by Anderson *et al.*[7] and Maxwell *et al.*[10] with the following principal exceptions: the present procedure employs a higher concentration of UDPGlu and a higher ratio of Gal-1-P to UDPGlu, less hemolyzate, and a shorter incubation period. A fourfold excess of Gal-1-P over that of UDPGlu was necessary for optimum transferase activity. While using these assay conditions, namely, 0.20 μM UDPGlu and 0.80 μM Gal-1-P, the amount of hemolyzate (equal volume of water and red cells) was varied in a 15-min incubation period to determine the optimum enzyme concentration. In similar experiments, the substrate concentration and the incubation time were varied while the amount of hemolyzate was held constant at 0.2 ml (Fig. 1). Reasonably good linearity was achieved until the

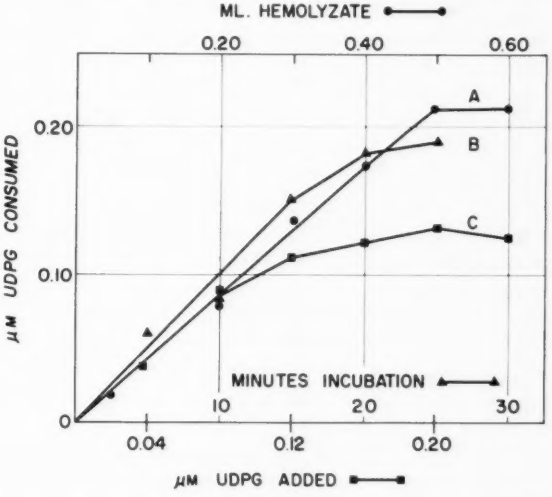

FIG. 1.—Three separate experiments are shown. In *A*, the volume of hemolyzate was varied and all other conditions were held constant. In *B*, the time and in *C*, the substrate level was varied. The following are the reaction conditions unless otherwise indicated: 0.10 ml of 1 *M* glycine, pH 8.7; 0.20 μM UDPGlu and 0.8 μM Gal-1-P (always added in a 1:4 ratio); and 0.20 ml. hemolyzate. Incubation time was 15 min.

UDPGlu became limiting at approximately 0.50 ml hemolyzate (curve A) or at 20 min incubation time (curve B). In the presence of excess substrate (more than 0.12 μM UDPGlu), incubation time and/or hemolyzate concentration became limiting (curve C). The difference in slopes of these 3 curves resulted from using different blood samples for each of the three experiments. For routine determinations, a 10-min incubation period and 0.20 ml of hemolyzate were selected, affording a wide range of measurable values with the same assay conditions. Adequate care was taken to insure excess of substrate for any given sample (usually 0.20 μM UDPGlu and 0.80 μM Gal-1-P).

The spectrophotometric comparisons were carried out in a Beckman DU spectro-

photometer with a photomultiplier attachment and a pinhole filter. The microcuvettes contained 0.20 ml DPN (0.10 μM) and 0.01 ml filtrate from the incubation mixture (omitted in control cell), final volume being made to 0.48 ml with glycine buffer (0.1 *M*, pH 8.7). Having the control cell set at zero absorption, the absorption at 340 mμ of the assay cell was recorded. UDPGlu dehydrogenase (0.01 ml) was then added to the control cell and reset to a zero absorption before a similar addition to each assay cell. After the increase in optical density had ceased, 0.01 ml of UDPGal-4-epimerase was added to each cell in the same manner as described for the dehydrogenase and any further reduction of DPN was noted. The difference in optical density increases before the addition of the epimerase was calculated as UDPGlu consumed in the transferase catalyzed reaction. The increase in optical density after addition of epimerase was calculated as UDPGal formed in the same reaction. The UDPGal recovered was 85 to 105 per cent of the UDPGlu consumed.

Results and Discussion.—In the application of the method, the levels of the transferase in red cells of galactosemics, their families, and normal individuals were determined. Results for three typical families and appropriate controls are shown (Table 1). A more detailed genetic study involving several more families will be published elsewhere. Enzyme levels are expressed as μM UDPGlu consumed per hour of incubation both per milliliter of red cells and per gram of hemoglobin. Before positive identification of the subjects, the values were categorized as follows: 0, galactosemics; 2.9–3.7, parents or carriers; 4.8–8.1, normal. Subsequent work with a greater population will define the expected range of transferase levels for patients, carriers, and normals more precisely.

Anderson *et al.*[7] reported normal transferase levels in the range of 2.4–3.5 μM of UDPGlu utilized per gram of hemoglobin per hour. The levels reported by Hsia *et al.*[9] are also in the same low category and consequently, when applied to a study of galactosemics and their families, there was marked overlapping in the various categories listed above, particularly in the carrier and normal groups. This is in part due to limitations of the assay employed since the high values were underestimated. The more quantitative procedure described herein yields values of greater magnitude, thus extending the over-all range with no overlapping between galactosemics, carriers, and normals. The carrier category is intermediate between the other two groups.

It has been suggested that galactosemia is inherited as a simple recessive characteristic.[6] Data presented here substantiate this concept. The results of the application of an improved enzymatic method to a study of transferase levels indicate that it is possible to detect parents who are likely to give birth to galactosemics and to survey a larger population of galactosemic and normal individuals to determine more precisely the genetics and also the incidence of this metabolic disorder. As galactosemia and other metabolic diseases may in some cases limit mental development, a consideration of transferase levels in mentally retarded patients should reveal if a significant number of patients have an abnormal galactose metabolism. Such a study is presently in progress.

Conclusions.—Existing procedures have been modified to determine galactose-1-phosphate uridyl transferase quantitatively in human erythrocytes. This method has been applied to galactosemics, their families, and appropriate normal individuals

TABLE I

GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE LEVELS IN RED CELLS

		μM UDPGlu Consumed per Hour	
		Per Ml Red Cells	Per Gm Hb
Family 1	Galactosemic	0	0
	Mother	2.9	8.7
	Father	3.5	10.3
	Sister	3.0	9.0
	Brother	7.0	21.2
	Sister	6.0	...
Family 2	Galactosemic	0	0
	Mother	3.7	10.8
	Father	3.6	10.7
Family 3	Galactosemic	0	0
	Mother	3.0	9.4
	Father	3.6	10.7
Controls			
9	Child—male	7.6	23.0
10	Child—male	6.9	21.5
11	Adult—male	8.1	25.4
12	Adult—female	5.9	18.8
25	Adult—male	6.0	17.7
51	Adult—female	6.0	18.3
52	Adult—female	5.0	14.9
53	Child—female	6.5	21.7
58	Child—male	5.0	15.8
60	Adult—male	4.8	14.7
61	Adult—female	5.0	14.7
Mean	Galactosemics	0	0
	Carriers	3.33	9.94
	Normal	6.14	19.0
Range	Galactosemics	0	0
	Carriers	2.9–3.7	8.7–10.8
	Normal	4.8–8.1	14.7–25.4

Incubation conditions were as follows: 0.20 μM UDPGlu, 0.80 μM Gal-1-P, 0.10 ml glycine buffer (1 *M*, pH 8.7), and 0.20 ml hemolyzate (1:1 ratio of red cells and water), made to final volume of 0.45 ml with water. Incubation time was 10 min at 37°C. The incubation mixture was denatured by placing in a boiling water bath for 2 min. After centrifugation, UDPGlu and UDPGal were determined as described in the text.

Results for three typical families are reported. As parents have a transferase level which is intermediate between the normal and the galactosemic, carriers of this genetic disorder may be detected by determination of transferase in the red cells. This procedure should be useful in a more detailed consideration of the genetics of this disorder.

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*THE SEDIMENTATION PROPERTIES OF THE LARGE SUBUNIT OF DNA RELEASED FROM LABELED T_4 BACTERIOPHAGE**

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Labeled T_2 and T_4 bacteriophage can be disrupted by a variety of procedures to release the encapsulated DNA into the medium. When P^{32}-labeled[1] phage are so disrupted and the unseparated reaction products examined by autoradiography,[2] one finds two classes of "stars" arising from decaying P^{32} atoms incorporated into the DNA: big ones with many rays and little ones with few rays. The "big stars" arise from particles which have 36 per cent of the total phosphorus of the phage. This large subunit (LSU) would correspond to a DNA molecule of 45×10^6 molecular weight. The "small stars" would correspond to DNA molecules of less than 12×10^6.[3] The LSU has been of interest because of the possibility that this particle is a viral chromosome.[4–7] Recent studies on the stability of the LSU to enzymes, heat, and P^{32} decay do indicate the LSU is a duplex DNA molecule unencumbered by any significant amounts of protein.[3]

In the present communication we wish to report the sedimentation coefficient of the LSU and show that the magnitude of this coefficient is in accord with expectation for a single DNA molecule containing 36 per cent of the total phosphorus of the phage as derived from autoradiography.

To determine the sedimentation constant of the LSU, the separation cells of Tiselius, *et al.*[8] and Yphantis and Waugh[9] were employed. To check the method, P^{32}-labeled phage were sedimented for 30 min at 9431 rpm in the Spinco Analytical Ultracentrifuge using standard procedures.[11] In some experiments 10^{11} carrier phage/ml. were added. Assays consisted of measuring the phage titer, the radioactivity, and the number of autoradiographic stars arising from intact phage, in the upper compartment, both before and after sedimentation. As sedimentation proceeds, the particles move out of the upper compartment. The Tiselius or fixed partition cell gave consistent results independent of carrier phage concentration while the movable partition cell of Yphantis gave erratic results. Therefore we report only the results taken with the fixed partition cell.

To interpret the data quantitatively one can either assume stable sedimentation with a well-defined boundary, independent of dilution, or one can imagine that at great dilution convective mixing occurs resulting from poor speed control and the absence of any stabilizing effect stemming from density gradients across the boundary. The expression relating the change in concentration Δc in the upper chamber with the sedimentation constant S and the time of sedimentation in seconds t for stable sedimentation is:[3]

$$1 - \gamma \frac{\Delta c}{c_0} = e^{-2\omega^2 St}, \tag{1}$$

where ω is the angular velocity, Co the starting concentration, and γ a geometrical constant. $\gamma = [r_b^2 - r_m^2[/r_b^2$, where r_b and r_m are the radii to the porous barrier and to the meniscus. Assuming *complete* convective mixing it can be shown that

the concentration in the upper compartment decays exponentially with time as follows:

$$C/C_0 = e^{-2\omega^2 St/\gamma} \tag{2}$$

Using phage titer, radioactivity, and star number, and by assuming stable sedimentation, we calculate for intact T_4, $S = 600$ to 800. This is independent of concentration down to 10^7 phage/ml. To fit the data with equation (2) which assumes connective mixing, would require a high value of $S = 1{,}450$ which does not agree with the optical determination. Therefore it is more reasonable to interpret the data in terms of stable sedimentation.

We turn now to the sedimentation of the LSU. Freshly prepared phage were purified by differential centrifugation until more than 90 per cent of the P^{32} label could be demonstrated to be in phage. The phage were osmotically shocked and the unseparated reaction product containing about 10^7 phage equivalents/ml were centrifuged as described above for 26 to 70 min at 25,980 rpm. Stars arising from the LSU's were counted and a graph of the results plotted in accordance with equation (1) is shown in Figure 1. From this graph one can calculate an average S value of 41 ± 5 for the LSU. Convection could not alter this estimate to a value greater than 58S and probably plays a much smaller role for the reasons outlined above.

The sedimentation coefficient for purified ghosts (the empty protein capsule of the phage) which were prepared by the method of Herriott,[10] was determined

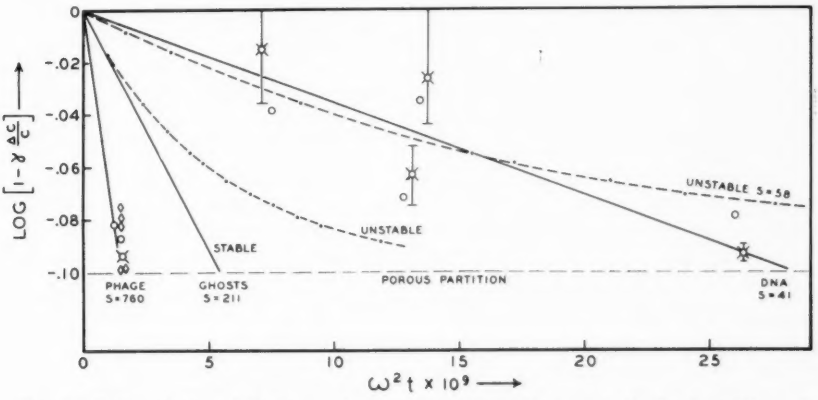

FIG. 1.—Sedimentation of the large subunit of DNA from T4 phage in the fixed partition cell. This graph displays the results of sedimenting intact phage (10^7/ml and 10^{11}/ml) and the osmotic shock product (10^7 phage equivalents/ml) dissolved in 0.10 M NaCl + 0.10 M CsCl + 0.032 M phosphate buffer pH 7.2, in the fixed partition cell. The undisrupted phage were assayed by phage titer (◈), by total P^{32} counts (⊙), or by number of stars formed by autoradiography (⊛). The shock product was assayed by total counts and by number of stars arising from the LSU. The error flags are drawn to indicate the standard error based on counting error alone. As sedimentation proceeds (as $\omega^2 t$ increases) the number of sedimenting particles in the upper chamber is depleted. This is revealed as a decrease in log $[1 - \gamma(\Delta C/C_0)]$. In the case of phage, the optical determination is shown by the solid line. In the case of the shock product, the points are fitted either by a solid line assuming stable sedimentation or a dotted line supposing complete confective mixing of the upper chamber. The expected sedimentation characteristics of protein ghosts produced by osmotic shock are shown for both the stable and unstable case, based on a value of $S = 211$ obtained from independent optical experiments.

optically at 10^{12} ghosts/ml. This value (211*S*) is shown in Figure 1. The 5-fold difference between this value and the sedimentation constant determined for the LSU makes it appear unlikely that the LSU is physically joined to the ghost protein.

Doty *et al.*[12] have studied a series of protein-free calf thymus DNA samples of different molecular weight that were prepared by sonication, a procedure which apparently snaps both polynucleotide chains at the same place. By independent sedimentation and molecular weight determinations they arrive at the following empirical relation between the sedimentation constant S and M the molecular weight:

$$S = .063M^{0.37}. \tag{3}$$

If one substitutes for M a value of $45 \pm 4 \times 10^6$, which is calculated from the average number of rays per star arising from the LSU, then one predicts a sedimentation constant of 41 to 44 *S*.

Thus we see good agreement between the *S* value obtained from these sedimentation experiments and that predicted by Doty's equation. This agreement would suggest that the LSU is hydrodynamically similar to protein-free thymus DNA which has been the subject of intensive physical-chemical and crystallographic study.[13]

Summary.—The sedimentation constant of the large subunit of DNA (LSU) which can be released from P^{32}-labeled bacteriophage has been determined. The value of the constant (41*S*) is just the magnitude expected for a DNA molecule at 45×10^6 molecular weight.

The authors wish to express their appreciation to Dr. Sol Goodgal for many helpful discussions and to James Brown for his valuable assistance.

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[1] Labeling is achieved by infecting bacteria which are growing in a medium containing P^{32} at a specific activity of 20 to 200 mc/mg and approximately 2γ inorganic phosphorus per ml.

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*THE PHYSIOLOGY OF ETHYLENE FORMATION IN APPLES**

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Communicated January 14, 1959

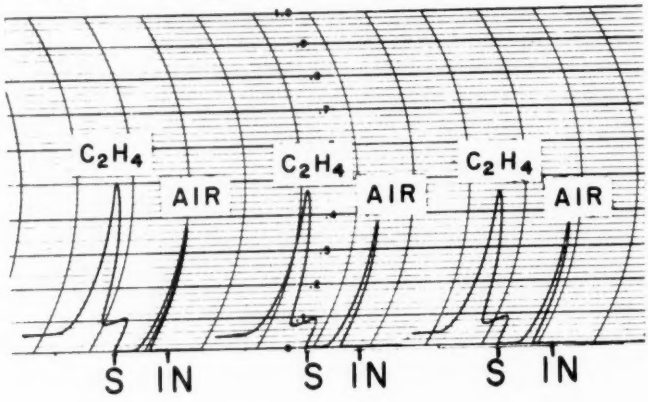
Introduction.—The toxic action of illuminating gas on plants has been known for nearly a century, and the fact that ethylene is the effective constituent was proved in 1901.[1] The effects of ethylene include inhibition of growth, loss of geotropic sensitivity, onset of epinastic curvatures, acceleration of respiration, initiation of rooting, modification of pigments both in leaves and in fruits, and hastening of fruit maturation. The discovery that ethylene can itself be produced by ripening fruits[2, 3] gives even greater interest to these phenomena, since it appears that the gas can in some circumstances function as an endogenous growth-controlling factor, or in fruits as a "ripening hormone." However, although there have been numerous studies on the action of ethylene, little is known about its formation. In large part this is due to the fact that it is produced in excessively minute quantities—measured in microliters per hour per kilogram of tissue—and existing methods are inadequate to follow its production with the precision and speed needed for physiological studies.

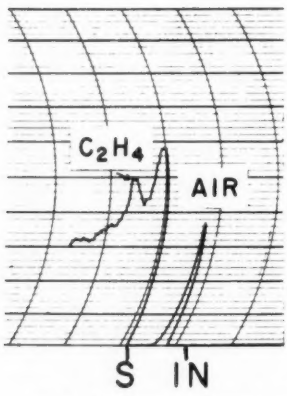
The advent of modern methods of gas chromatography has made possible a new approach to the study of ethylene formation in biological materials. In the experiments of which the present paper is a preliminary report, this approach has led to the development of a method about 1,000 times more sensitive than the assays which have been employed in the past. Some applications of this methodology to the problem of the biogenesis of ethylene will be described.[4]

Method and Materials.—In brief, the method consists of injecting the gas sample into the end of an adsorption column consisting of 18 in. of Tygon tubing packed with aluminum oxide. The column is developed with helium, and the issuing gases detected and measured by a high sensitivity katharometer.[5] Several features adopted in the construction of the katharometer combine to produce very nearly the theoretical limit of sensitivity which is obtainable by the thermal conductivity principle. The inherent noise level of the instrument was minimized by using filaments which had been spot welded to the lead-in wires; the filaments of the katharometer were run at the temperature (80°C) which gave the highest signal to noise ratio; the output signal was magnified with an amplifier which had a lower noise level than the katharometer when the latter was run at its optimal wire temperature; the signal produced for a given change in thermal conductivity was maximized by using very fine, high resistance coiled filaments suspended in long, narrow channels. In this way a combination of high sensitivity and low noise level could be achieved which allowed the detection of quantities of ethylene below 5×10^{-3} μL, while the chromatographic column permitted the use of total gas volumes as large as 2 ml. By comparison, the manometric method generally used,[6] even if modified by reducing the volume of absorbent to a minimum, cannot very well detect less than 10 μL of ethylene.

Figure 1 illustrates results obtained with the procedure outlined above, demonstrating its consistency, sensitivity, and ability to separate closely related com-

pounds. For any one constituent, the peak height was found to be linearly related to the total volume of gas present throughout the range 10^{-3} to 10^{3} μL. The relation between peak height and quantity was determined for each gas by empirical calibration. Samples containing apple emanations characteristically separated





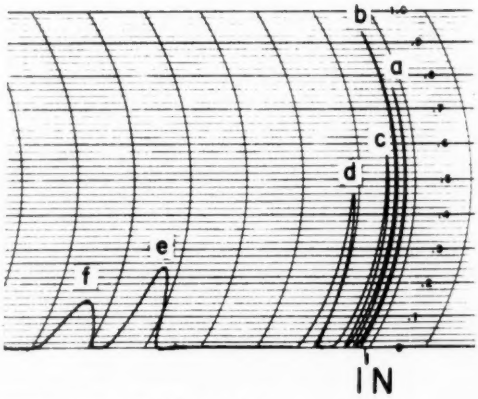


FIG. 1.—Performance curves. (Each record reads from right to left.)

Top: Three successive gas chromatograms of approximately 0.5 ml samples of air which were withdrawn from a Baldwin apple with a syringe. At the point marked *S*, the sensitivity of the analyzer was increased 1,950×. Vertical lines on the graph are spaced at 1-min intervals. Values for the percentage ethylene in the internal atmosphere calculated from the three peaks are 0.163, 0.160, and 0.163, respectively.

Lower left: A gas chromatogram of 0.5 ml of air containing 6.8×10^{-3} μL of ethylene, i.e., about 13 ppm. The sensitivity was increased 58,000× at the point marked *S*, and thereafter the full scale corresponds to 1.0 μV. Vertical lines on the graph are spaced at 1-min intervals. A correction must be applied for the air "tail" preceding the sample reading, and for a slow battery drift which occurs at very high sensitivities.

Lower right: A fractionation of 1.5 ml of natural gas to which 0.25 ml of ethylene has been added The peaks are (*a*) methane, (*b*) ethane, (*c*) ethylene, (*d*) propane, (*e*) isobutane, and (*f*) *n*-butane. The sensitivity was increased 33.2× between *a* and *b*, reduced 7.3× between *b* and *c*, increased 535× between *c* and *d*, and increased 4,000× between *d* and *e*. Vertical lines on this graph are spaced at 4-min intervals.

into two fractions on the aluminum oxide column: a large first band containing oxygen, nitrogen, carbon dioxide, and perhaps trace amounts of other volatiles, and a small second band containing only ethylene.

McIntosh apples, purchased at the local market and stored in polyethylene containers at 4°C, were used in all experiments. When the apples were transferred from cold storage to room temperature, they consistently showed a maximum rate of ethylene production after 10 hr and were used at this time. About a dozen plugs were removed from a single apple with a No. 5 cork borer; these were cut to 3.8 cm length, rinsed in tap water, dried, and weighed (*ca* 2.7 g each). The sections were then inserted in 5 ml hypodermic syringes, the bore of these syringes being just sufficient to accommodate the tissue. When the plunger of a syringe was set at 4.2 ml, less than 2 ml of air was present within the tissue and free air space. The actual air content was calculated from the facts that apple tissue has a density of 0.8,[7] and a free air space of 33 per cent.[8] Needles were attached and sealed with rubber stoppers; the tissue was then incubated for 60 min, after which time the stoppers were removed and the contents of each syringe pressed out. Apple tissue is soft enough to pass through the needle, and the entire gaseous contents were collected over water (the solubility of ethylene in water is so low that the concentration of the gas is essentially unchanged by this procedure). A gas sample was then drawn up in a 2-ml syringe and chromatographed. The rate of ethylene production was calculated from the total air and ethylene recorded in the analysis, and the initial air volume in the syringe, weight of tissue, and total time of incubation. Preliminary experiments showed that apple plugs extracted from various parts of the fruit have the same rates of ethylene production per gram within ±5 per cent, and this rate is the same as that of the whole fruit from which the sections were cut. The production rate of the whole apple was determined by a somewhat different procedure,[9] which need not be detailed here.

Syringes containing apple tissue were exposed to various gas mixtures by placing them tip downward in a desiccator, evacuating the system, and running a known gas mixture into the desiccator. Water was then admitted through a separatory funnel fixed in the top of the desiccator, and the excess pressure which developed was relieved through an overflow. When the tips of the syringes were covered with water, the desiccator lid was removed, and the syringe needles were added and stoppered under water.

Respiration rates of apple plugs were determined in constant pressure respirometers,[10] a differential system with and without alkali being used for carbon dioxide measurements.

Counting of tritium-labeled ethylene was carried out in the gas phase after extensive purification by condensation and redistillation.[9]

Results.—(*1*) *Effect of evacuation:* Since apples may contain large amounts of ethylene in their intercellular spaces, measurements of the rate of production can only be made after this gas has been removed; otherwise it might represent a considerable portion of the ethylene present after a short collection time. An experiment was therefore devised to ascertain whether evacuation had any effect on the subsequent rate of ethylene production. Total ethylene content was determined on unevacuated plugs after 30 and 90 min, and the production rate was calculated by subtracting the 30-min from the 90-min total. A second set of syringes, con-

TABLE 1

EFFECT OF EVACUATION ON ETHYLENE PRODUCTION BY MCINTOSH APPLE TISSUE SECTIONS

Time, Min	Ethylene, $\mu L/Kg$	
	Unevacuated	Evacuated
30	159.0	...
60	...	133.0
90	288.8	...
120	...	269.6
Rate; $\mu l/Kg/hr$	129.8*	134.8 av.
C_2H_4 initially present ($\mu l/Kg$) calculated	94.1	0

* Ninety-minute total minus 30-min total.

taining duplicate samples from the same apple, was evacuated in a desiccator, and production of ethylene was determined after 60 and 120 min. The data in Table 1 show that the evacuated tissue maintained a constant rate of ethylene production of about 135 $\mu L/Kg/hr$ for the 2-hr period, whereas the unevacuated tissue—although it initially contained about 94 $\mu L/Kg$ of ethylene—produced 130 $\mu L/Kg$ in a 1-hr period (between 30 and 90 min). Evacuation, therefore, has no effect on the subsequent rate of ethylene production. This fact also strongly suggests that none of the precursors of ethylene is volatile. In all further experiments the tissue was evacuated just before the syringes were sealed.

(*2*) *Temperature dependence:* The influence of temperature was investigated by incubating syringes containing tissue from a single apple in various temperature-controlled water baths. Figure 2 summarizes the results obtained. Over a 2-hr period, the optimal temperature was found to be 32°C, and the Q_{10} between 10 and 25°C was 2.8. Over the same temperature range, the Q_{10} values for oxygen consumption and carbon dioxide production were found to be 2.75 and 2.5 respectively; (the carbon dioxide production of the tissue declined during the 2-hr

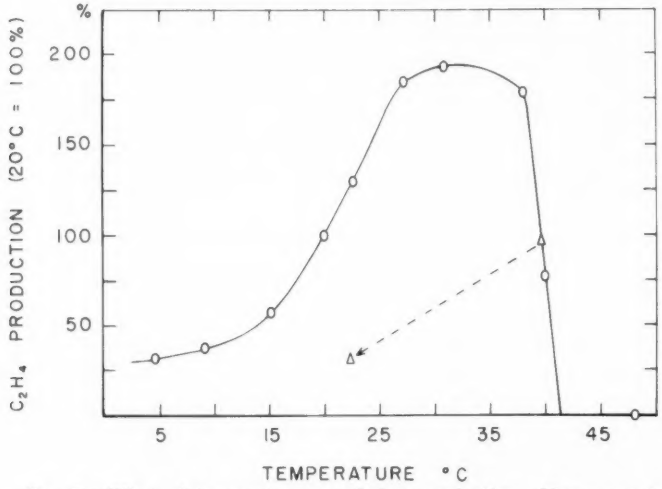


FIG. 2.—Effect of temperature on ethylene production. Measurements were made over a 2-hr period, each point being the average of many experiments. The value at 20°C was arbitrarily selected as 100 per cent. Triangles indicate the behavior of tissue held at 40° C for 1 hr and then replaced at 22°C for the next hour.

period, so the Q_{10} for this process is not exact). At 40°C and at higher temperatures, ethylene production is severely inhibited, and tissue held at 40°C for 1 hr produces little ethylene during a subsequent hour at a lower temperature (see Fig. 2). However, the temperature inhibition is not irreversible, and after 5 hr at room temperature about 50 per cent of the temperature-induced inhibition usually disappears. Hansen[11] observed a similar recovery of ethylene production after pears had been heat treated.

(3) *Effects of nitrogen, oxygen, and carbon dioxide:* Several workers have reported that ethylene production ceases under anaerobic conditions[2, 11, 12]. The effect of reduced oxygen partial pressure on the ethylene production and oxygen consumption of apple tissue plugs is shown in Figure 3. The points at the lower oxygen tensions are subject to some uncertainty because of the difficulty of making precise correction for the amount of oxygen consumed during the 1 hr experimental period. However, the data show clearly that the effect of oxygen tension on ethylene production is either identical with, or similar to, its effect on respiration. This suggests either that both processes donate electrons to the same terminal oxidase, or else that the production of ethylene is dependent upon aerobic respiration for energy or for substrate. Since the apple shows a strong Pasteur effect, it is unlikely that the tissue energy supply would be sufficiently depleted under anaerobic conditions to stop ethylene production completely. On the other hand,

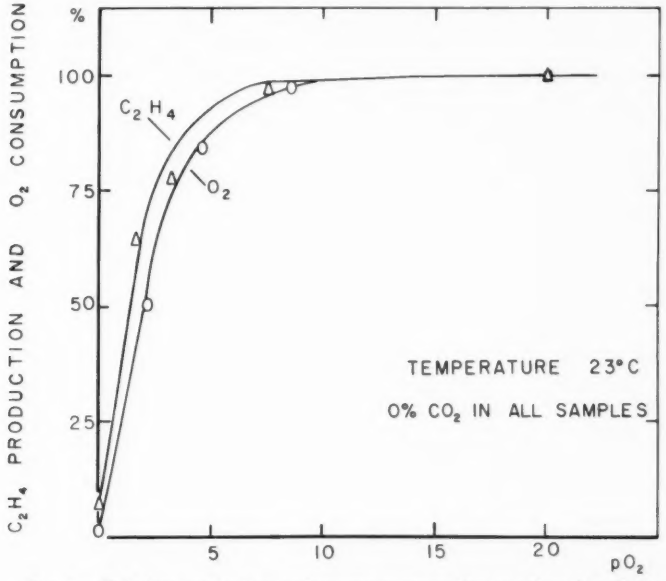


FIG. 3.—Ethylene production and oxygen consumption as a function of pO_2. Measurements were made over a 1-hr period after the gas mixture had been vacuum-impregnated into McIntosh apple tissue sections. All mixtures contained only O_2 and N_2; control tissue was treated with 80 per cent N_2 — 20 per cent O_2. The rates of both ethylene production and oxygen consumption of the controls are represented as 100 per cent. The ethylene curve (triangles) has not been corrected for the small ethylene production which occurs in the absence of O_2.

ethylene is produced only during the maturation period when apple tissue has a cyanide-insensitive respiration, and ethylene production has been found to be unaffected by cyanide.[9] The evidence at hand supports the idea that a cyanide-insensitive terminal oxidase of relatively high oxygen affinity is involved both in respiration and in ethylene production.

The data in Figure 3 show that there is a very slight production of ethylene during a 1-hr exposure to anaerobic conditions. The time course of this production was studied by placing plugs of tissue in syringes, evacuating to about 1 mm pressure, and then admitting nitrogen. Results are shown in Figure 4, curve *A*. A very small amount of ethylene is made for about 15 min after oxygen has been removed; then production of the gas ceases indefinitely. Since the preparations for this experiment required at most 2 min, the curve cannot be displaced more than 1 or 2 min along its axis from the exact moment at which anaerobic conditions were obtained. Although the small trace of ethylene produced after the onset of anaerobiosis might be accounted for by residual oxygen in the tissue, it is unlikely that very much oxygen remained and other evidence will be cited which supports the view that this ethylene was actually produced in the absence of oxygen.

Despite the fact that ethylene production soon ceases in nitrogen, some other reactions preliminary to ethylene formation apparently continue anaerobically

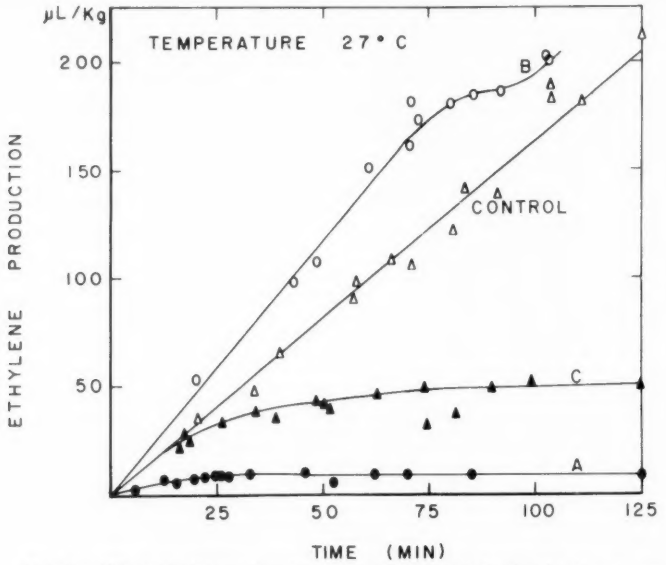


FIG. 4.—Effect of nitrogen treatment on ethylene production by tissue sections. The values on the graph are average figures compiled from many experiments with McIntosh apple tissue sections. Control curve: production of ethylene by sections kept under atmospheric conditions for 4 hr before experiment. Curve *A:* ethylene production immediately after tissue was placed under nitrogen. Curve *B:* ethylene production by tissue previously stored under nitrogen for 4 hr, and then replaced in air. Curve *C:* in these experiments the tissue had been stored in nitrogen for 4 hr, placed in air for 5 min, re-evacuated, and returned to nitrogen, after which the ethylene production was recorded.

at a substantial rate. Thus, apple tissue which had been allowed to remain under nitrogen for 4 hr showed an accelerated rate of ethylene production when it was evacuated and the nitrogen replaced by air (Fig. 4, curve *B*). In Figure 4, the control curve represents the behavior of sections which had been cut from the same apple and allowed to remain in room air for 4 hr before being tested. A few apple plugs were assayed immediately after removal from the fruit, and they showed the same rate of ethylene production as the tissue used in deriving the control curve. Hence the high rate of ethylene production displayed by nitrogen-treated tissue is not the result of a decreased control rate. It should be noted that this increase in rate does not occur after the removal of some other inhibitors. For instance, high concentrations of carbon dioxide will retard ethylene production, an 80 per cent CO_2–20 per cent O_2 mixture giving about 40 per cent inhibition. However, after 4 hr, when the mixture is replaced with 20 per cent O_2–80 per cent N_2 the rate of ethylene production immediately returns to the control level, but not above it. It appears that prolonged nitrogen treatment causes the accumulation of some material (presumably a reduced compound), which can be converted to ethylene very rapidly in the presence of oxygen.

This same type of behavior is exhibited by whole apples. The internal concentration of ethylene in an apple can be readily determined by extracting a small sample of air from the fruit with a hypodermic syringe; this sample can then be gas-chromatographed. If the extraction is carried out under water, considerable accuracy can be obtained (as for example in Fig. 1, top). When whole apples were evacuated in a desiccator, and replaced in air, the internal ethylene accumulated at a decreasing rate as shown in Figure 5, *Controls*. When the apples were re-evacu-

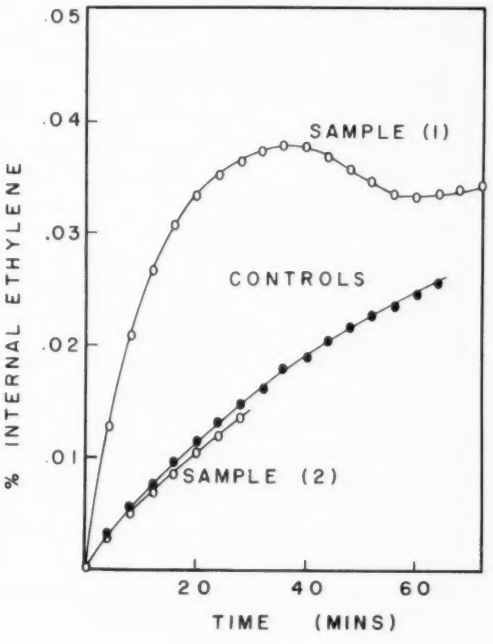


FIG. 5.—Effect of nitrogen treatment on ethylene production by whole apples. When McIntosh apples were evacuated and returned to atmospheric conditions, the internal ethylene concentration increased along the curve shown as Controls. The fruits were re-evacuated at the last point indicated on this curve, after which the internal ethylene accumulated along an identical curve. Other apples were vacuum-impregnated with nitrogen and left for 4 hr. The fruits were then evacuated and placed in air, after which the internal ethylene concentration rose along the curve marked Sample 1. When the tissue was re-evacuated after 65 min, the internal concentration now increased at the same rate as the control, Sample 2. Each curve is an average compiled from the behavior of three apples. Four hours after the last recorded points, the samples had reached an internal concentration of 0.0471 per cent, and the controls 0.0572 per cent.

ated, the ethylene again accumulated along an identical curve. The curve marked *Controls* in Figure 5 actually comprises points from before and after the re-evacuation. However, if the apples had been stored under nitrogen for 4 hr and then evacuated and returned to atmospheric conditions, a remarkably high rate of ethylene accumulation resulted (Fig. 5, *Sample 1*). When, after 65 min, the treated apples were re-evacuated, they then showed a low rate of ethylene accumulation equal to that of the controls (Fig. 5, *Sample 2*). This experiment has been carried out three times with virtually identical results. It is evident that nitrogen storage leads to an accelerated rate of ethylene production when aerobic conditions are subsequently reinstated.

The experiments shown in Figures 4 and 5 indicate that the initial acceleration of ethylene production occurring after nitrogen storage is short-lived, and it is followed by a transient period in which little or no ethylene is produced. In Figure 4 the decline takes place at about 75 min, when the rate falls essentially to zero before it recovers to what appears to be a control value. In Figure 5, after about 30 min the internal concentration of ethylene begins to fall in *Sample 1*, before it again rises at about 55 min. The discrepancy in timing between the whole fruit and tissue plugs is not understood, but in general the results show that some precursor can be converted anaerobically to a substance which is oxidized to ethylene, that this precursor becomes depleted in nitrogen, and that it is replaced only slowly in air.

Even if apple tissue which has been in nitrogen for 4 hr is exposed to air for only 5 min, it can go on to produce ethylene for about 60 min when it is replaced under anaerobic conditions. The initial rate of ethylene production of tissue so treated, as shown in Figure 4, curve *C*, must be about the same as in control tissue. This supports the preceding conclusion that a substance accumulates under nitrogen, and it can then be oxidatively converted to ethylene. In addition, curve *C* indicates that the terminal step or steps in ethylene production must be nonoxidative. The oxidative "reaction" must be extremely rapid since it can supply sufficient oxidized precursor in 5 min to support subsequent ethylene production for about 60 min. Normally, the concentration of the oxidized precursor present in the tissue must be so low that it can be exhausted within 15 min in the absence of oxygen (see Fig. 4, curve *A*).

(*4*) *Experiments with labeled precursors:* Experiments with carbon and tritium labeled compounds will be described in a future publication, but a few results are included here because of their relationship to the scheme of ethylene production which can be derived from the experiments cited above.

If tritiated water is admitted into apple tissue, its label becomes incorporated very rapidly into the ethylene. Since the dissolving of ethylene in tritiated water was found to produce little or no exchange, it follows that the incorporation into ethylene in the tissue is metabolic. In the same period of time which is required for complete equilibration of the added isotope with the tissue water (about 3 min), the ethylene was found to reach approximately the same specific activity as the isotopic water within the tissue. Water must therefore be able to enter and leave one or more precursors of ethylene in the position which will ultimately correspond to the ethylenic double bond.

The role of reducing hydrogens in ethylene synthesis was studied by means of tritiated glycerol. The isotope contained a nonlabile-tritium in the 2-carbon posi-

tion; this is the position known to yield reducing hydrogen when glycerol is metabolically dehydrogenated.[13] The glycerol was soaked into large plugs of apple tissue for 60 min under anaerobic conditions in order to prevent its metabolism during the feeding period.[14, 15] After the sections had been exposed to air for a further 15 min, a significant transfer of label to malic and succinic acids and sugars had occurred; in one instance the ethylene was also radioactive but somewhat low in specific activity, while in a second experiment no measurable activity could be detected in the ethylene at all. Malic acid, which represents a large metabolic pool in apple tissue, has three nonlabile hydrogens and two of these are in a position where known reactions would result in an extensive dilution with water. Yet this substance had a higher specific activity (620 cpm/μM) than the ethylene (0 and 550 cpm/μM), even though the latter compound is present in minute amounts and contains four nonlabile hydrogens. It follows that, in the synthesis of ethylene, if reductions (involving hydrogen atoms which can be even indirectly derived from the 2-carbon position of glycerol) occur, they must take place prior to the hydration-dehydration reaction indicated in the preceding paragraph.

Discussion.—The data presented in the preceding sections are summarized in Figure 6 in the form of a sequence of reactions leading to the production of ethylene. The evidence for the various steps is as follows:

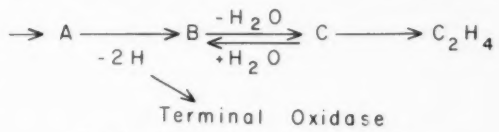


FIG. 6.—Scheme for ethylene production.

1. Compound *A* accumulates under nitrogen, by one or more reactions probably involving reductions. The existence of this substance is revealed by the accelerated rate of ethylene production observed when oxygen is readmitted (Figs. 4 and 5).

2. The almost immediate cessation of ethylene production under anaerobic conditions indicates that ethylene production is in some way linked to the utilization of atmospheric oxygen. Although ethylene production and oxygen uptake show a very similar dependence upon oxygen partial pressure (Fig. 3), it is unlikely that the correlation is mediated through energy production. In the case of ethylene, a cyanide-insensitive terminal oxidase may accept electrons derived from a dehydrogenation step, here pictured as the conversion of *A* to *B*. The same terminal oxidase may also accept electrons from respiration.

3. Evidence for a reversible dehydration step which cannot be followed by a hydrogenation or reversible dehydrogenation, was derived from experiments with tritiated water and tritiated glycerol. This is represented as the conversion of compound *B* to *C*.

4. The final step or steps in ethylene production must be nonoxidative since tissue treated with nitrogen for 4 hr is able to produce ethylene anaerobically after a brief exposure to oxygen (Fig. 4, curve *C*). The oxidative conversion of *A* to *B* must be extremely rapid, for when the short oxygen treatment is terminated, a sufficient quantity of oxidized precursor has been produced to sustain ethylene production for a considerable time.

5. It should be noted that *A*, *B*, and *C* are all nonvolatile.

Summary.—The production of ethylene by sections of apple tissue has been

investigated by a new and precise method. The optimal temperature for the process is 32°C, and above this ethylene production falls off rapidly. The heat inactivation slowly disappears when the tissue is exposed to lower temperatures. Ethylene production and oxygen consumption show almost identical dependence upon oxygen tension, the half maximum value for both being reached at 1.5–2.0 per cent O_2. Although the synthesis of ethylene ceases almost immediately under anaerobic conditions, a precursor accumulates which can be rapidly oxidized in air with the production of ethylene. Tritiated water transfers its label to ethylene in such a manner as to indicate that one of the terminal steps in the process is a reversible dehydration. The final reaction leading to ethylene does not require the participation of oxygen. A scheme for ethylene production is proposed in which one or more reductions are followed by a rapid oxidation, a reversible dehydration, and a nonoxidative terminal step.

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PHOTOCONTROL OF PLANT DEVELOPMENT BY THE SIMULTANEOUS EXCITATIONS OF TWO INTERCONVERTIBLE PIGMENTS

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Flowering, seed germination, stem elongation, and many other aspects of growth and development of seed plants are influenced by a reversible photoreaction.[1, 2] This reaction can be written:

$$PH_2 + A \underset{7{,}350\ \text{A max}}{\overset{6{,}600\ \text{A max}}{\rightleftharpoons}} P + AH_2$$

in which PH_2 and P are the reduced and oxidized forms of a pigment with absorption maxima near 6,600 and 7,350 A, respectively, and A and AH_2 are a hydrogen acceptor and donor. The oxidized pigment, P, is an enzyme controlling an essential reaction.

Interconversion of the oxidized and reduced forms of the pigment adequate to produce striking biological responses can be achieved with low irradiances. In other words, because of the high absorption coefficients, these being greater than 10^7 cm^2/mol as measured by the reversibility of the photoreaction,[3] change in the form of the pigment can readily be affected. An example is the response of imbibed lettuce seeds to an alternation of low irradiances in the region of 6,600 and 7,350 A, respectively. Germination of the seeds is suppressed or potentiated depending upon the final exposure.[1] In a more quantitative way, an incident energy of 10^{-7} einsteins/cm^2 (1.54×10^4 ergs/cm^2) in the region of 7,350 A can diminish P to $1/e$ of its initial value when the quantum efficiency is forced toward 1.0 by a high ratio of $[AH_2]$ to $[A] + [AH_2]$, and an incident energy of 10^5 ergs/cm^2 under these conditions can diminish P to 0.15 per cent of its initial value.

TABLE 1

EFFECTS OF EXPOSURE TO SUNLIGHT ON GERMINATION OF *L. virginicum* SEEDS

Exposure to Full Sunlight, sec	Germination with Prior Treatment	
	Red Radiation, %	Darkness, %
0	64	0
1	42	21
2	51	27
4	53	29
8	85	30
16	87	36
32	93	48
64	93	62

Plants under high irradiances in the region of 6,000 to 8,000 A show responses arising other than from the reversibility of the preceding photoreaction. Thus cocklebur (*Xanthium pensylvanicum*) growing on short days and long nights fails to flower if the night is interrupted near the middle with a low irradiation of 5×10^4 ergs/cm^2 in the region of 6,600 A in the order of 1 min. They are readily reinduced to flower if this irradiance is followed by an equivalent one in the region of 7,350 A. If the irradiance in the region of 7,350 A is greater than the order of 10^6 ergs/cm^2, however, the plants fail to flower. The high incident energy in the region of 7,350 A produces a biological response similar to that in the region of 6,600 A. The effects with high irradiances are now found to arise from the continued photoexcitation of the pigment in those spectral regions where both the oxidized and reduced forms have appreciable absorptions. In the further excitation, reactions other than the reversible one occur which, at least, change the ration of [A] to $[AH_2] + [A]$ by inducing reactions of A and AH_2 with reactants other than the pigments. The phenomenon warrants further explanation before experimental results are presented.

Absorbances of the oxidized and reduced pigment in the region of 5,800 to 8,000 A are shown in Figure 1, *C*. The solid lines are derived from measurements of reversibility of specific responses and the courses of the dotted extensions are obtained from the present work. A crossover or isobastic point exists near 6,950 A. Under continuous irradiation in this region both pigment forms are excited. Since

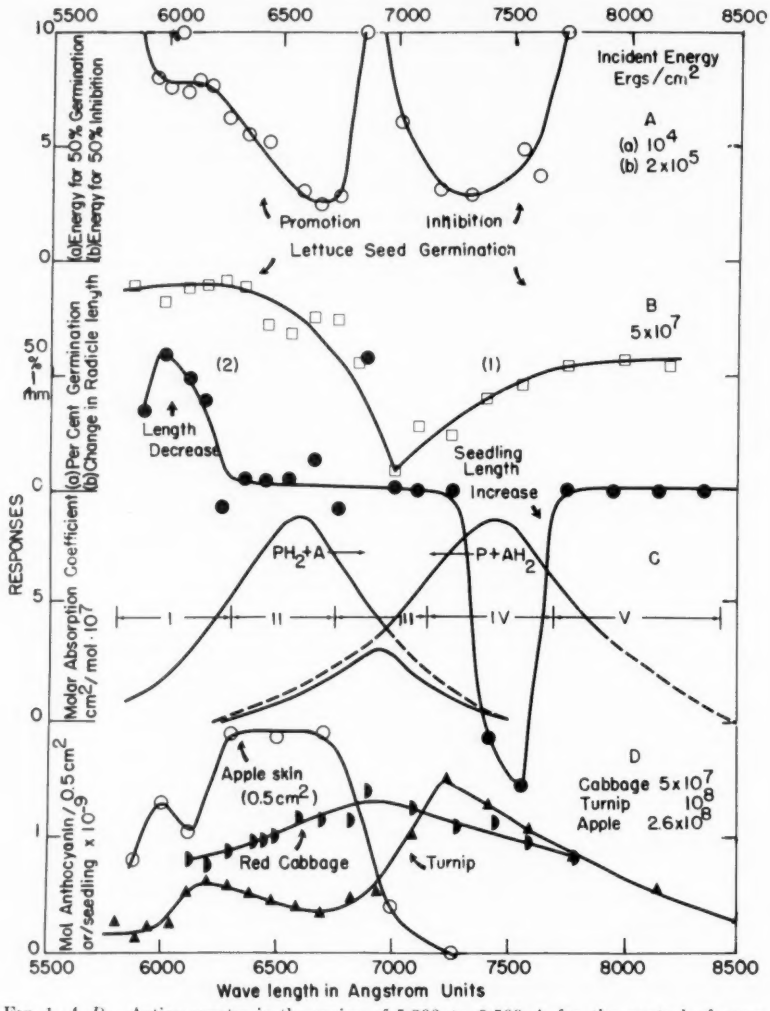

FIG. 1, *A–D*.—Action spectra in the region of 5,800 to 8,500 A for the control of some aspects of plant development. Note text for explanation.

these are photochemically effective pigments, they can take part in reactions other than the reversal. The probability of repeated excitation, because of the possibility of reversal, depends upon the absorbancies of both forms of the pigment in any given region as well as upon the ratio of the oxidant to the reductant in the photoreaction. Thus, if the concentrations of A and AH_2 are about equal, the maximum of an action can be near the isobastic point, as shown by the lowest curve in Figure 1, *C*, instead of near the absorption maxima.

The absorptions in wave-length regions longer than 5,800 A can be grouped into

five limiting regions which intergrade as the limits are approached. These regions are:

I. 5,800–6,300 A: Absorption of radiation is due very predominantly to PH_2, thus driving the photoconversion with high irradiance very far toward P.

II. 6,300–6,700 A: Includes the absorption maximum of PH_2 and accordingly requires the least irradiance for conversion of PH_2 to P. The absorption of P is increasing in this region and in the steady state the amounts of both P and PH_2 are appreciable, with P predominant.

III. 6,700–7,200 A: The region of the isobastic point with the maximum of the action near 6,950 A. Amounts of P and PH_2 are of the same order of magnitude provided the concentrations of A and AH_2 are about equal.

IV. 7,200–7,800 A: Includes the absorption maximum of P as well as appreciable absorption of PH_2.

V. >7,800 A: Absorption of radiation due very predominantly to P thus driving the photoreaction very predominantly to PH_2. The absorbancy, however, is small but can still be detected by biological response at wave lengths above 7,800 A.

Action is also evident at wave lengths shorter than 5,800 A with a maximum effectiveness near 4,500 A. Effects in this region have not been fully disentangled.

Action spectra for a number of growth and development responses compel the conclusions and illustrate the Classes I to V as well as the effects of various degrees of oxidation of A. Photocontrol of anthocyanin formation is recognized as a response to be considered, although it previously had not been realized as arising from action of the reversible pigment system. A first example is the action spectrum for anthocyanin production in seedlings of red cabbage (*Brassica oleracea* var. Red Acre)[4] shown in Figure 1, *D*. It has the features of the combined action in region III with a maximum near 6,900 A. The promotion and inhibition with low irradiances of lettuce seed germination (var. Grand Rapids)[1] shown in Figure 1, *A* illustrate Classes II and IV.

An example of all five classes is afforded by the response to high irradiances of seeds of the Great Lakes variety of lettuce, which germinate fully in darkness at 23° and are not inhibited by low irradiances in the region of 7,350 A. Imbibed seeds were held in darkness at 23° for 16 hr after which time some had germinated. They were then irradiated for 12 hr on a two-prism spectrograph with an energy flux of 0.2 milliwatt/cm^2, a dispersion of 70 A/cm, and a slit width of 170 A at 7,000 A. They were returned to darkness for 12 hr after which germination and radicle length of germinated seeds were measured with results as shown in Figure 1, *B*. The action maximum and shape of the action curve for germination are in accord with the action in region III. In particular, supression of germination is detectable near 6,600 where the absorbancy of PH_2 is maximum. The suppression is maximum near 7,000 A and decreases to longer wave-lengths where the absorbancy of P is maximum.

The action spectrum for radicle length of Great Lakes lettuce shown in Figure 1, *B* was most unexpected, and, in fact, was the initial basis for the findings reported here. The elongation is suppressed with a maximum near 6,100 A and is enhanced with a maximum near 7,500 A. There is very little response elsewhere. The suppression of elongation is in region I and is at considerably shorter wave lengths than the maximum absorbancy of PH_2. The enhancement is displaced to longer

wave lengths than the absorbancy maximum of P. The apparent lack of effect of radiation in the region of 6,300 to 7,200 A might well result from the balancing of several actions.

The action spectrum for anthocyanin formation in apple skin[5] (Fig. 1, *D*) corresponds to regions I and II. There is no doubt about the experimental presence of the subsidiary maximum in the region of 6,000 A corresponding to the maximum suppression of radicle elongation for lettuce. The most effective region for anthocyanin formation is II, which probably results from a very great predominance of AH_2. Thus, the red absorbing form, PH_2, of the pigment can be repeatedly excited without being converted to the far red absorbing form, P. The synthesis of anthocyanin apparently is limited by the excitation of PH_2. The apple skin also produces ethanol aerobically, which could well be associated with the extreme reduction of A to AH_2.

Further features of the combined action of the two pigment forms are evident in the action curve for anthocyanin synthesis in turnip seedlings (*Brassica rapa* var. Purple Top White Globe) inhibited with chloroamphenicol to prevent chlorophyll development. The greater portion of this curve is identical in shape with the germination curve for Great Lakes lettuce seed (Fig. 1 *B*) expressed as effectiveness of irradiance for germination instead of inhibition as shown. The secondary maximum near 6,100 A corresponds to the maximum for suppression of elongation of the lettuce seedling radicles. In the turnip seedlings A must be greatly predominant over AH_2. As a result, the maximum for action is near the absorption maximum for P, the far red absorbing form of the pigment in contrast to results for apple skin. In the region of maximum absorption of PH_2 anthocyanin synthesis is suppressed by excitation of PH_2 with essential quenching of the excitation of P for formation of anthocyanin.

Anthocyanin syntheses in the three objects, apple skins, and red cabbage and turnip seedlings, while having greatly different action spectra are examples of Classes II, III, and IV, respectively. All depend to a marked degree upon simultaneous excitation of PH_2 and P, but the apple skin is driven chiefly by excitation of PH_2, the red cabbage seedlings by PH_2 and P, and the turnip seedlings by P. Two of these three have the subsidiary maximum near 6,100 A corresponding to the predominant conversion of PH_2 to P, with P being effective in the dark synthesis of anthocyanin as is known for red cabbage seedlings.[4]

Further comments about the photoreaction might be illustrative. Pigments P and PH_2 can be associated with either A or AH_2 leading to activation upon photoexcitation. However, when P is associated with A, excitation is ineffective for conversion to PH_2 and similarly for PH_2 associated with AH_2 to be converted to P. The lifetime of excited P and PH_2 would be expected to be enhanced under these conditions. Thus, A and AH_2 could be excited by energy transfer to react with another hydrogen donor or acceptor. The ratio of [A] and [AH_2] to [AH_2] + [A] would be changed by such a reaction in which an alternative hydrogenation could be affected as, for example, one in anthocyanin synthesis. A biological test can be made for the change in ratio of [A] to [AH_2] + [A]; namely, the sensitivity of the photoreaction in the two directions can be measured, an increased rate of PH_2 reacting with A under constant irradiance corresponding to an increase in the [A] to [AH_2] + [A] ratio. An example of a change of this type is the response of

Lepidium virginicum seeds to continued irradiation with sunlight as a source of high irradiance after a short irradiance in the region of 6,600 A adequate to potentiate full germination. Results shown in Table 1 indicated that after 1-sec exposure to sunlight the potential germination is at a minimum which is shifted to full germination in 64 sec, supposedly by the conversion of AH_2 to A.

The many evident implications of these findings to the photoperiodic control of flowering and other aspects of plant growth and development will not be discussed. But the fact that anthocyanin synthesis is responsive to photoexcitation of the reversible pigment system merits emphasis. It permits, for instance, estimation of the concentration of the pigment in the cells in which anthocyanin synthesis takes place. This dependence opens the phenomenon to biochemical examination in that the pertinent chemistry is in the utilization of acetyl, both in aromatic synthesis (by still unknown pathways)[6] and in fat metabolism.

Summary.—The pigment in the reversible photoreaction controlling plant growth and development at low irradiances is effective at high irradiances in spectral regions where the absorbancies of the oxidized and reduced pigment form are both appreciable. The action with high irradiance depends in part upon the simultaneous absorptions of the two forms. This action controls anthocyanin formation and many other responses including some aspects of photoperiodic induction of flowering and seed germination.

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*EXTENSIVE DEEP SEA SUB-BOTTOM REFLECTIONS IDENTIFIED AS WHITE ASH**

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Communicated by Maurice Ewing, January 2, 1959

Introduction.—Sub-bottom echoes have been observed since the advent of expanded scale recording sounders. Since the introduction of the Precision Depth Recorder (PDR) with a short ping (about 5 millisec) sub-bottom reflections have been recorded at many locations in the deep sea, e.g., Heezen *et al.*[1] The observed sub-bottom reflections have not been systematically cored. On *Vema* cruise 15 (V-15) such an echo was observed over a very large area of the eastern part of the

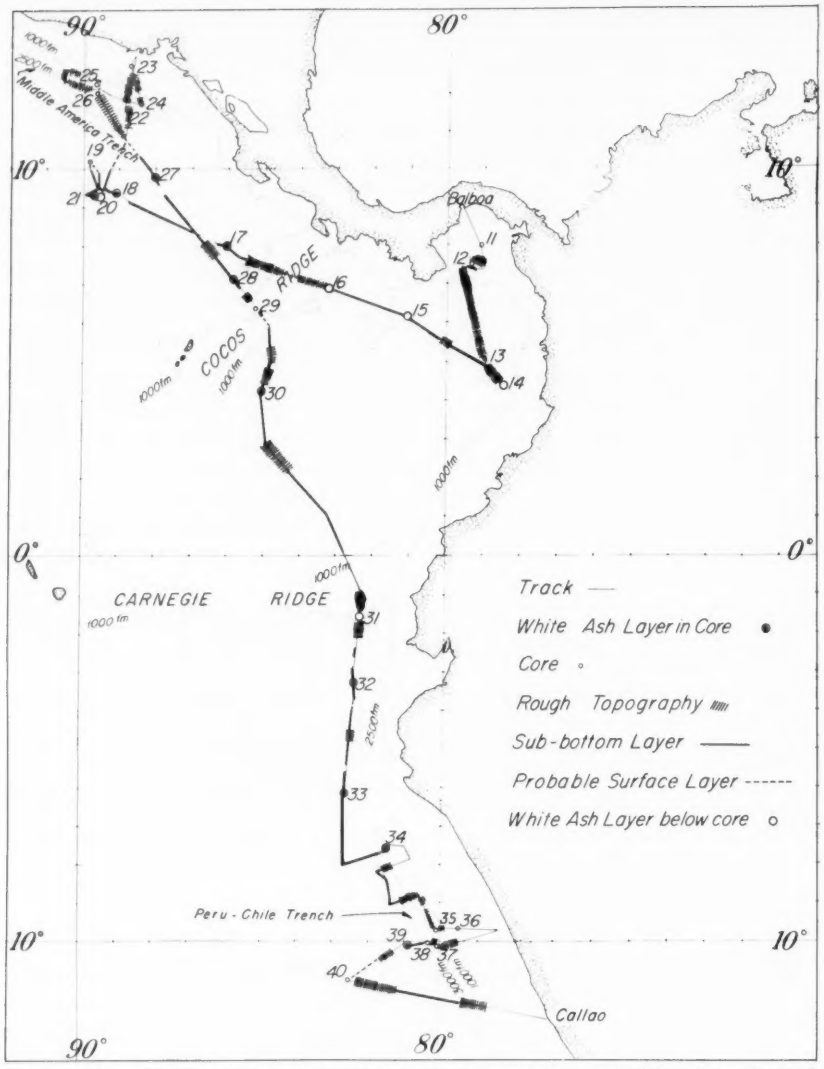

FIG. 1.—Chart of *Vema* Cruise 15 showing core locations and type of PDR echoes obtained.

tropical Pacific Ocean. Long piston cores at eight locations penetrated to the depth of this echo in the sediment, and in most cases well beyond. A layer of clean white ash was encountered in each of these cores at the appropriate depth. The description of cores in this report is based on preliminary shipboard examination.

Instrumentation.—The PDR is a high resolution sounder utilizing a precision time recorder for depth determination in the deep ocean. A 12-kc pulse of 5 millisec duration is emitted at 1-sec intervals in a cone of approximately 15° directed

at the bottom from a keel mounted transducer. The returning echo is timed by a precision frequency source and recorded on a continuous paper strip on a scale such that 400 fm is represented on the full paper width of 19 in. (43 cm). The time is converted to fathoms of depth using the convenient 4,800 fps sound velocity. A more detailed description of the PDR was given by Luskin *et al.*[2, 3]

The piston-coring device consists of a 2.5-in (6.25-cm.) steel pipe up to 80 ft (2,438 cm) or more in length to which is attached a 1,200-lb (545 kilos) lead weight. The core tube and weight are supported on a triggering device attached to a 0.5-in (1.27-cm.) wire rope. The triggering device is arranged with a trigger weight which releases the corer so that it has a free fall of 10 ft (305 cm) before striking the ocean bottom. The 0.5-in. wire rope is so arranged that it permits the free fall of 10 ft and is connected to a piston located initially at the bottom end of the core tube. The winch operator stops the winch when he detects the change of tension in the wire rope caused by the release of the core weight. If all adjustments have been properly made, the piston is arrested at the ocean floor while the core weight and tube continues down to sample the ocean floor. The piston serves as a device to overcome the friction of the sediment on the internal walls of the coring tube. We were able to take cores of up to 78 ft (2,377 cm) in length in this area on V-15.

Description of Data.—Figure 1 shows part of the track of V-15 under consideration. *Vema* left Panama on November 13, 1958, and landed at Callao, Peru, on December 13, 1958. The 1,000-, 2,500-, and 3,000-fm contours were taken from the General Bathymetric Chart of the Oceans[4] modified according to the work of Heacock *et al.*,[5] Shumway,[6] and Zeigler *et al.*[7]

A heavy line represents the track where a clear sub-bottom echo is present. Occasionally two and rarely three sub-bottom echoes can be observed. Unfortunately we were never able to obtain a core sufficiently long to be able to identify the deeper sub-bottom echoes. Along parts of the track, the sub-bottom reflection rose until it could not be resolved from the bottom echo (about 1 fm). A dashed line indicates such sections of the track. The track is cross-hatched where the bottom is so rough that sub-bottom reflections can be confused with side echoes so that identification on the PDR records is uncertain. In many of these areas a sub-bottom echo is almost certainly present in the valley bottoms and is strongly suggested on slopes and hill tops. The track is indicated by a narrow line where no sub-bottom echo was observed.

Coring locations are shown on Figure 1 by circles with the core number written alongside. In cores 17, 18, 21, 22, 27, 28, 30, 32, 33, 34, and 39, a white ash layer of 2.5 to 30 cm was encountered. These circles are double. In cores 17, 18, 22, 28, 32, and 33 these white ash layers correlate well (see Table 1) with the sub-bottom echoes indicated on the PDR records. In cores 21 and 27 the white ash layer occurs so close to the bottom that it could not be resolved as a separate echo on the PDR record. Examples of PDR records may be seen in Figure 2. The PDR record has interference from a scattering layer at 150 to 200 fm obscuring the bottom echoes at the time of core 30. The sub-bottom echo observed on the PDR is below the depth penetrated by cores 14, 15, 16, 20, and 31. These cores are indicated by large open circles in Figure 1. In core 34, the white ash is only 3 cm thick. A very faint sub-bottom echo appears to be present on the PDR record but it is questionable. The coring equipment operated improperly for cores

TABLE 1

WHITE ASH OCCURRENCE IN CORES AS COMPARED TO SUB-BOTTOM ECHOES ON THE PDR

Core No.	Longitude	Latitude	PDR Depth, fm	Core Length, cm	White Thickness, cm	Ash Depth, cm	Sub-bottom Echo Depth, cm	Remarks
11	79-03W	07-55N	133	409	..	...	...	
12	79-21	07-29	776	906	..	...	Rough	
13	79-04	05-00	1,670	1,125	..	...	Rough	
14	78-27	04-25	2,008	1,019	..	...	3,840	
15	81-03	06-08	1,428	1,196	..	...	2,743	
16	83-16	06-53	1,218	1,224	..	...	2,195	
17	86-00	07-55	1,650	1,704	15	305	366	
18	89-05	09-20	1,868	1,930	10	366	366	
19	89-50	10-07.5	1,970	541			Surface	(Piston jammed)
20	89-32	09-18	1,870	No core recovered			366	(Trigger fouled)
21	89-39	09-20.5	1,857	2,383	10	00	Surface	
22	88-43	11-35	2,071	1,097	8	213	183	
							3,292	
							3,840	
23	88-37	12-35	150	521	..	...	...	
24	88-25	11-40	2,972	1,077	..	...	...	(White ash chunks present at 640 cm)
25	89-43	12-18	2,475	1,123	..	...	...	
26	89-34	12-00	2,480	859	..	...	*	(White ash chunks present at 610 cm)
27	88-00	09-43	1,700	2,296	10	21	*	(White ash chunks present at 488 cm)
					8	366		
28	85-55	07-05	1,535	1,975	30	366	360	
29	85-17	06-21	1,015	1,180	..	...	*	(White ash chunks present at 61 cm)
30	85-06	04-15	1,750	1,238	3	335	†	
					3	610		
31	82-19	01-30S	760	610	..	...	914	
32	82-30	03-15	1,529	1,186	10	640	640	
33	82-41	06-18	2,158	1,308	8	1,036	1,097	
34	81-24	07-35	3,092	1,153	3	701	732?	(Very weak sub-bottom echo)
35	80-04	09-43	1,540	1,034	..	...	*	
36	79-28	09-40	95	1	..	...	...	(Only a handful of shells)
37	80-09	10-12	3,340	1,246	..	...	366	(Series of thin layers 305–366 cm in
38	80-19	10-02	2,990	1,659	..	...	†	core)
39	80-54	10-12	2,545	1,513	6	30	*	
40	82-31	11-05	2,380	1,730	..	...	Rough	

* Cannot resolve individual echoes from broad distributed echo.
† Scattering layer interference.

19 and 20 and such cores as were recovered may not have sampled all of the layers present. At these stations short gravity cores were taken, which have not yet been examined. The PDR echo at the time of cores 26 and 35 was a broad distributed echo in which no individual echoes could be resolved. No ash layer was found in either core 26 or 35, but both were located on walls of deep sea trenches. In the remaining cores, no ash layer was found and no sub-bottom echoes were found on the PDR records to indicate such layers should be present.

As may be seen (Fig. 2) when the white ash layer was present in thicknesses greater than 5 cm the sub-bottom echo is much stronger than the bottom echo, and has a duration of approximately that of the outgoing ping. The strongest sub-bottom echo (Fig. 2, *B*) is obtained in core 28 where the layer is 30 cm thick.

With the exception of cores 11 and 36, which were taken on the continental shelf, and cores 31 and 40, all the cores showed a progressive change from a dark olive green medium firm clay to a light olive green medium firm clay from top to bottom. Besides the white ash layer, there were occasionally very thin black ash layers. Core 29, taken on the Cocos Ridge, apparently contained a clay breccia. A sample of the white ash layer was brought back to the laboratory and has been identified by David B. Ericson as a very fine-grained ash of clear glassy fragments, many of which are needle-like. A sample of the green clay also examined contains foraminifera, clay, and a finer-grained fraction of ash similar to the white ash. The green color is probably imparted by organic remains. Core 31 was brown to green foraminiferal sand throughout, indicating strong winnowing by bottom currents. This is probably characteristic of the crest of the Carnegie Ridge, and accounts for the absence of the ash layer and of the sub-bottom reflection.

Core 39, located near the sill depth of the Peru-Chile trench, showed the white ash layer only 6 cm deep. Alternating layers of black, brown, gray, and green clays form most of the section below the ash, suggesting that the section has been thinned by periodic slumping or scouring.

Core 40 consisted of gray clay from the top to 450 cm, at which point it grades to tan. Below 900 cm it is dark tan. At 640 cm, a 7.5-cm. hard black brittle fine-grained layer (probably manganese) was found. Because the topography is somewhat rougher in this area, it was difficult to distinguish between sub-bottom and side echoes at the time the coring tube hit. However, unmistakable sub-bottom echoes were recorded from the gentler slopes in the area. The compaction of the lower part of the core suggests pre-Quarternary age. Since this core was clearly on a steep slope, the question of disturbance or alteration of the ash layer must remain open. Further detailed examination of the cores will have to await their return to the laboratory in a few months.

Discussion.—The great extent of a sub-bottom echo at depths of 0 to 40 meters below bottom in the tropical Eastern Pacific has been demonstrated. Occasionally there are two, and rarely three, sub-bottom echoes. The first sub-bottom echo is well correlated by cores throughout the area with a white ash layer. Since the layer is fairly near the surface and is not discolored and contains nothing but the glassy ash material, it must have been laid down fairly quickly. Foraminifera in the clays above and below the layer promise that a date can be obtained after detailed examination of the cores. It is important to further delineate the area from which this strong sub-bottom echo is obtained and to sample the layer to

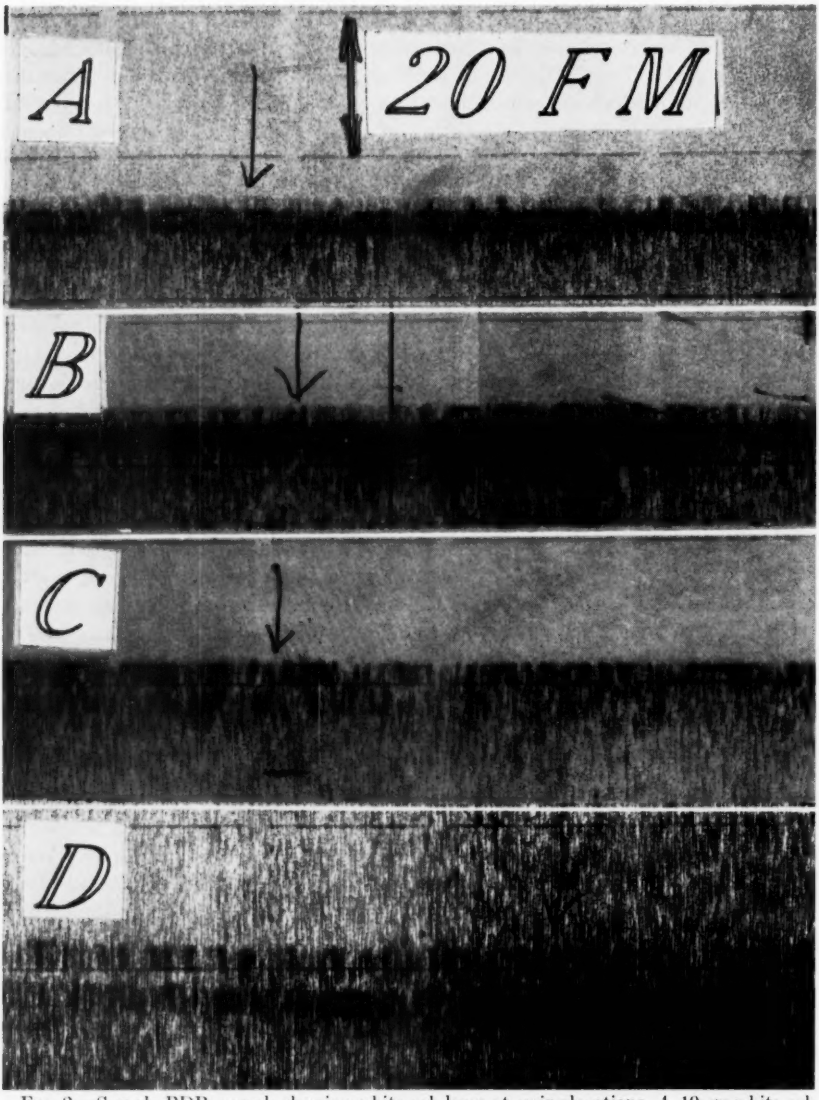

FIG. 2.—Sample PDR records showing white ash layer at coring locations. *A*, 10-cm white ash layer 1.8 fm (329 cm) deep in core 18. Water depth 1,868 fm. *B*, 30-cm white ash layer 2.0 fm (365 cm) deep in core 28. Water depth 1,535 fm. *C*, 10-cm white ash layer at surface in core 21. Water depth 1,857 fm. *D*, 8-cm white ash layer 5.7 fm (1,043 cm) deep in core 33. Water depth 2,158 fm.

determine whether it is due to the same white ash layer found here. The layer rises to the ocean floor toward the higher parts of the topography and deepens in the lower parts. It disappears on the shelves. It cannot be certainly identified in rough topography such as the walls of the Middle America and the Peru-Chile

Trenches, but it is almost certainly present in the valley bottoms. It is not found on the trench floors, perhaps due to its deep burial.

If the white ash layer can be identified with the same time of occurrence in all of the cores, much can be learned of the sedimentation rates in this part of the Pacific and particularly about the variability of these rates.

The white ash immediately suggests a volcanic origin and the proximity of the Andes suggests the source. However, the great extent of the ash and its shallow cover would imply such a great amount of recent activity for a short time that it may well be difficult to ascribe it to the Andes. Until its extent, age, and identification are determined one can only speculate. Perhaps sub-bottom echoes from other areas can also be correlated with this white ash layer. If so, it may be necessary to attribute the layer to a world-wide volcanism or perhaps to the fiery end of bodies of cosmic origin.

It will be possible to measure the acoustic properties of the clays above and below the layer and of the layer itself. This area can then become an excellent testing ground for theories of reflectivity for various layer thicknesses and depth of burial and for the effectiveness of various sounding equipments.

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* Lamont Geological Observatory Contribution No. 337.

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*SIGNIFICANCE OF THE WORZEL DEEP SEA ASH**

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Introduction.—In the preceding communication Worzel has described a bed of white ash which he discovered in deep sea cores and was able to trace over a large area in the Pacific as a sub-bottom sound reflector. While following the sub-bottom echo, he was able to take additional cores to prove the correlation between the ash

layer and the reflector. Worzel shows that the ash extends at least from 11° North to 12° South Latitude within a few hundred miles of the coasts of Central and South America. A single ash layer of 5 to 30 cm thickness over such a wide area must record a notable event in the history of the area. It could hardly be without some recorded consequences of global extent. The following is a preliminary note, based on shipboard descriptions of the cores, calling attention to other data relevant to Worzel's discovery. The writers hope that other investigators will join them in determining the properties and extent of this layer.

Sub-bottom Reflectors.—Sub-bottom reflectors have been observed since the first self-recording echo sounders were put into use. However, sub-bottom echoes were rarely recorded in water depths greater than 300 fm because the compressed scales of most deep water recorders severely limited resolution. The Hughes echo sounder of the *Albatross* and *Galathea* was provided an expanded scale suitable for observation of deep water sub-bottom echoes.[1] Deep water sub-bottom echoes were not observed by American workers until the development of the Precision Depth Recorder in 1953.[2] Sub-bottom reflections obtained with sounders operating at 10 or 12 kc/sec have been identified only rarely by sediment coring. Heezen *et al.*[3] correlated a prominent sub-bottom echo in the Cariaco Trench with the base of the Recent anaerobic layer. In shallow water, Smith,[4] Moore,[5] and others have identified bed rock, the base of the Recent, and other horizons. However, Worzel has made the first successful identification of a sub-bottom reflector in very deep water. Koczy[1] has published the echograms of the round-the-world *Albatross* expedition. Examination of the photographic reproductions of these echograms reveals the existence of sub-bottom echoes in many of the areas traversed. In certain areas sub-bottom echoes are observed as much as 25 fm below the ocean floor. Heezen, Tharp, and Ewing[6] have described sub-bottom reflections observed in the North Atlantic.

Sub-bottom reflections in shallow water, in restricted basins, from abyssal plains, and in the lee of topographic highs can be related to local causes: turbidity currents, unconformities, and so forth. However, sub-bottom reflections over gentle rises isolated from down-slope sediment movements must reflect events of wider importance. Thus, while we must be aware of a variety of other causes of reflections, it is conceivable that we may find that the Worzel ash extends well beyond the area in which it has been mapped.

A re-examination of the file of *Vema* echograms is now in progress. It shows that sub-bottom echoes, similar to those found in the eastern Pacific, have also been recorded in the south Atlantic and Indian Oceans. In general, the reflector was so deep that it was not reached by cores, but in the Gulf of Mexico, correlation with an ash layer in some cases and with a carbonate layer in others (Ewing, Ericson, and Heezen,[7] pp. 1019 and 1021) is found. From the cores and echograms in our collection and from those to be taken during the present cruise of *Vema*, we hope to identify the reflectors in the areas traversed. Thus we should learn whether all reflectors in each area result from local causes or if some are of world-wide extent.

The intensity of a reflection from a thin layer of material having an impedance different from that of the surrounding sediment will be greatest if constructive interference occurs between the sound returning from the top and bottom of the

layer. Taking 1.60 gm/cm^3 and 1.60 km/sec for the green lutite and 2.00 gm/cm^3 and 2.40 km/sec for the ash, and neglecting rigidity, the fraction of the incident energy returned to the water from an ash layer when constructive interference occurs has been calculated by J. E. Nafe as 27 per cent, compared with 6 per cent for the bottom echo. Constructive interference would be expected at odd multiples of one-quarter wave length. However, with variations in layer thicknesses and deviations from plane parallel interfaces, the reflected energy would ordinarily be an appropriate average between the maximum and minimum of the order of 10 per cent of the incident energy. However, if the layer thickness falls below one-quarter wave length (about 5 cm at 12 kc) the reflected energy would drop rapidly, becoming less than the bottom reflection at thicknesses of 1 to 2 cm.

Preservation of Stratification.—The action of deep sea bottom currents, bottom burrowing organisms, and surficial gravity movements produces mechanical mixing of most sediments. The ash deposits observed by Kuenen and Neeb[8] and Bramlette and Bradley[9] were mixed through a column of sediments several times the thickness of the original ash bed. In addition to this mechanical mixing, solution may vastly alter the sediment before permanent burial is accomplished. Devitrification and alteration, proceeding at rates dependent on the environment, may transform an ash bed into products whose origin is not readily recognized.[10]

Beds have a greater chance of preservation when high rates of deposition occur. Broecker, Turekian, and Heezen[11] have shown that the productivity of foraminifera varies only slightly with time in the equatorial Atlantic. If this is a general characteristic of foraminiferal production, the low carbonate content of the sediments in the area of the Worzel ash indicates high rates of terrigeneous or organic sedimentation.

Ash Layers.—Extensive ash layers are now recognized in continental areas throughout the geological record (e.g., Ross,[12] p. 428). Frequently the ash was transported so far that its place of origin is in doubt. Until recently ash falling into the ocean was considered lost to the geological record. Murray and Renard[13] identified volcanic particles in practically all of the *Challenger* surficial samples of deep sea deposits, demonstrating that volcanic detritus is an important component of modern deep sea deposits throughout the world. They suggested that the abyssal clays are largely the result of alteration of volcanic ash. The recognition of beds of ash was impossible until core samples became available.

Correns[14] reported aeolian deposits of ash and desert quartz in the *Meteor*'s equatorial Atlantic cores.

Bramlette and Bradley[9] were able to trace two ash layers throughout most of the ten cores along Piggot's North Atlantic profile. According to the temperature estimates of Cushman and Henbest,[15] Plate IV, one of these layers appears to be at the beginning and the other at the end of the last cold interval represented in these cores. The refractive index is given as 1.51 for the glass of both layers.

Kuenen and Neeb[8] studied the distribution of ash from recent eruptions of Tambora and Oena Oena. The ash was observed in surficial samples out to 400 km and in some cases constituted 60 to 80 per cent of the sample out to distances of 300 km.

Arrhenius[16] found volcanic ash distributed in the east equatorial Pacific cores. In some cores the ash was bedded. Although sub-bottom echoes can be seen on

published *Albatross* echograms obtained in the general vicinity of these cores, no correlation can be made from the published data.

Mellis[17] described two beds of ash found in the *Albatross* Mediterranean cores (refractive indices 1.51 and 1.52) and attempted to correlate them with eruptions of Santorini. As for the Pacific cores mentioned above, echograms for some areas near the core stations[1] show sub-bottom echoes, but again no positive correlation is possible from the published data.

Norin[18] and Mellis[19] described ash derived from nearby Italian volcanoes in two cores from the Tyrrhenian Sea. Many of the beds were deposited by turbidity currents, hence dating and correlation with known eruptions are doubtful.

The ash-rich layer found in the Gulf of Mexico was at about the same depth as the Worzel ash in the Pacific and had a similar refractive index of 1.498.[7] It was similar to the ash of Piggot's North Atlantic cores in being dispersed in normal sediments and in its stratigraphic position near the beginning of the last cold period.

Zen[10] has made preliminary descriptions of a series of cores taken by the *Atlantis* near the coasts of Chile and Peru, between 15° and 25° South Latitude. He logs "abundant, partly devitrified glass" in most samples but in several cores, layers are found whose megascopic descriptions are "white or yellowish silty mud." These were found to contain abundant glass which, in one instance, is described as "fresh and colorless." Zeigler, Athearn, and Small,[20] in their brief report on the topographic work of the *Atlantis* expedition, make no mention of sub-bottom echoes, but in the reproduction of one echogram (17° South Latitude) a reflector about 20 m below bottom is clearly shown. This work is taken as an indication that the Worzel ash may be traceable to at least 25° South Latitude.

Probable source and distribution of the Worzel ash: The ash consists of colorless shards of volcanic glass with an index of refraction of 1.500 and varying in size from 0.07 to 0.2 mm. There is no particle size sorting. Most of the shards are in the form of curved, fluted, or crumpled films of glass. A minority are nearly equidimensional fragments of silky pumice. No crystalline minerals have been found. In all important respects it is similar to material which has been classified as volcanic ash in the deep-sea deposits of the world. On preliminary examination, the ash of the Worzel layer appears to be quite similar to the ash layer which occurs in a suite of cores from the Gulf of Mexico.[7]

Rex and Goldberg[21] have found quartz particles of continental origin in abundance in Pacific sediments as much as 2,000 miles from the nearest continent. They attribute this wide dispersion to atmospheric transportation. Since it is very possible that the shards of the Worzel layer were thrown to a great height in the atmosphere by an explosive eruption or eruptions, and since the shards because of their peculiar shape would settle more slowly in both air and water than nearly equidimensional quartz particles, an ocean-wide or even global distribution of the Worzel ash is a real possibility. The remarkable uniformity of thickness of the Worzel ash layer within the large area which has been cored is additional evidence suggesting that the layer may well have great areal extent.

The sediment in which the Worzel layer occurs is a greenish gray sticky lutite. Its color and general appearance when dry suggest that it contains a fairly large proportion of finely divided volcanic glass. Coccoliths and broken radiolaria and

diatoms are abundant in the fine fraction. Fully 90 per cent of the coarse fraction (>74 microns) consists of the tests of planktonic foraminifera. The presence of a few benthic foraminifera indicate that the bottom water where this sediment was deposited must have contained some oxygen. Other organic constituents are radiolaria to the extent of about 3 per cent of the coarse fraction and a few diatoms among which fragments of *Ethmodiscus sp.* are dominant. The coarse fraction also contains several per cent of colorless glass shards which are quite similar to and have the same index of refraction as those in the Worzel ash. The sample described here was scraped from the outside of a long core and consequently there is a possibility that it may have been contaminated by shards from the Worzel layer. However, the large proportion of shards in the coarse fraction render this explanation rather improbable.

The greenish sediment is probably deposited more rapidly than normal pelagic sediments. Therefore, the sediments covering the ash layer (mean thickness about 4 m) probably represent no more than 100,000 yr, and conceivably far less.

The ash is entirely unlike material described as meteoritic dust.[22] Only the wide geographic extent of this layer suggests any source other than volcanic eruptions.

There is abundant evidence of Quaternary andesitic volcanism along the entire length of the Cordillera, and the explosive volcanoes of Central America constitute one of the most important sites of Recent pyroclastic activity in the world. In the Galapagos Islands, Recent and Quaternary volcanic activity, principally pyroclastic, has been classified as "almost andesitic" by Banfield, Behre, and St. Clair.[23]

The majority of Andean volcanoes are probably too old to have provided the Worzel ash. However, as Lewis, Tchopp, and Marks[24] point out, El Sangay, El Sumaco, and El Reventador in eastern Ecuador are large cones, practically uneroded, "and certainly of Quaternary age."

Thus three possible volcanic sources for the ash are Galapagos, eastern Ecuador, and Central America. The outstanding difficulties are the wide extent and the uniqueness (in the cores of this area) of the ash layer, difficulties which are also encountered in the explanation of beds of bentonite.

The Worzel ash is apparently without regional trend in thickness over an area at least 2,500 km long and as much as 500 km wide, lying about equally in the northern and southern hemispheres. Prevailing surface winds throughout the area are easterly. The ocean currents are very complex in this equatorial region. Coastal currents converge on the area from both directions, joining the west-flowing North and South Equatorial Currents. The surficial Equatorial Counter Current and the newly discovered Cromwell Undercurrent[25] converge on the area from the west. Thus both atmospheric and marine dispersal are possible, but there is apparently no combination of these methods which could provide uniform ash thickness over area already surveyed without providing substantial thickness over a far larger area.

The descriptions of *Albatross* cores[16] do not mention discrete ash layers. This line of cores was 1,000 to 1,500 km west of the *Vema* track, in an area of eupelagic sedimentation, where accumulation is expected to be much slower. The possibilities that the ash layer was very thin, or that it has been altered, suggest that a re-examination of some of these cores might be profitable. The piston cores and the Precision Depth Recorder echograms from the recent Scripps Down Wind

expedition (Fisher[26]), should provide definitive data concerning the Worzel ash.

The extensive layer found in the Gulf of Mexico at about the same depth as the Worzel ash could be part of the same fall. As mentioned before, the essentially identical stratigraphic position of the ash in the Gulf of Mexico and the lower ash in Piggot's North Atlantic cores suggests that these are parts of a single fall. If so, the total volume must be much greater, and the mechanism of dispersal so effective that we should be able to extend the boundaries of the fall even farther. If this suggestive correlation can be proved, the total volume of ash must be so great and the mechanism of dispersal so effective that the possibility of world-wide coverage must be considered.

At approximately the same stratigraphic position, in the Equatorial Atlantic cores[27] a thin clay, superficially similar to bentonite and containing "a flood of diatoms," suggests an even greater extension of the fall. This material, although not definitely identified as ash, suggests the answer to the question of how submarine ash layers can possibly be so widespread as the Worzel ash without having been frequently identified in the past. It is necessary to study the alteration of fine pyroclastics in the sea and to set up criteria for recognition of the alteration products formed under the full range of environmental conditions.

Ash of similar composition has been logged in boreholes in many of the dry lakes of the western United States.[28] We cannot confirm or exclude the possibility that the ages are the same but faunal correlations are not available, and radiocarbon dates cannot be extrapolated safely in this type of deposit.

The question of whether this ash fall is really far more extensive than any hitherto known will probably receive a definite answer during the present cruise of *Vema*. The value of such a fall as a marker would be great, but such an event could hardly fail to produce a variety of significant effects, global in scale. Apparently we require either a single very large volcanic explosion, or the simultaneous explosion of many volcanoes, or conceivably a cometary collision similar to that suggested by Urey[29] as a "last resort type explanation" for the origin of tektites.

* Lamont Geological Observatory Contribution Number 335.

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ON THE NUMBER OF IRREDUCIBLE CHARACTERS OF FINITE GROUPS IN A GIVEN BLOCK

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Let $\mathfrak{G}$ be a group of finite order g and let p be a fixed prime number. The blocks of irreducible characters of $\mathfrak{G}$ with regard to p and their significance for the arithmetic in the group ring of $\mathfrak{G}$ have been discussed in several previous publications.[1] In particular, it has been stated that the number m of ordinary irreducible characters in a p-block of defect d is at most equal to $p^{d(d+1)/2}$. In the present note, this result will be improved. We shall show:

THEOREM 1. *Let B be a* p*-block of defect* d *of a finite group $\mathfrak{G}$. The number* m *of ordinary irreducible characters $\chi_1, \chi_2, \ldots \chi_m$ in B satisfies the inequality*

$$m \leq {}^1/_4 p^{2d} + 1. \tag{1}$$

Our new proof which we shall now sketch is far more elementary than the previous proof of the weaker result. Let B consist of the modular irreducible characters $\varphi_1, \varphi_2, \ldots \varphi_n$. For p-regular elements R of $\mathfrak{G}$, i.e., for elements R of $\mathfrak{G}$ whose order is prime to p, we have formulas

$$\chi_i(R) = \sum_{j=1}^{n} d_{ij}\varphi_j(R), \qquad (1 \leq i \leq m) \tag{2}$$

where the d_{ij} are nonnegative rational integers, the decomposition numbers of $\mathfrak{G}$ belonging to B. Set

$$a_{ij} = (p^d/g) \sum_R \chi_i(R)\overline{\chi_j(R)}, \qquad (1 \leq i, j \leq m) \tag{3}$$

where R ranges over all p-regular elements of $\mathfrak{G}$. Let A denote the $m \times m$ matrix (a_{ij}) and let D denote the $m \times n$ matrix (d_{ij}). If D' denotes the transpose of D, then $C = D'D$ is the $n \times n$ matrix of Cartan invariants of $\mathfrak{G}$ belonging to B. It follows from (2), (3), and the orthogonality relations for the modular group characters that

$$A = p^d DC^{-1}D'. \tag{4}$$

It is known that C has integral coefficients and that its largest elementary divisor is p^d. Now (4) shows that A has integral coefficients. Clearly, A is a symmetric matrix.

The nth determinantal divisor of the $m \times n$ matrix D is 1.[2] Using the normal form of D, we deduce from (4) that there exists an $m \times m$ matrix U with integral coefficients and determinant 1 such that

$$UAU' = p^d \begin{pmatrix} C^{-1} & 0 \\ 0 & 0 \end{pmatrix}.$$

It follows that not all coefficients of UAU' are divisible by p and hence not all coefficients of A are divisible by p.

Let $\{R_1, R_2, \ldots, R_h\}$ be a system of representatives for the classes of p-regular conjugate elements of $\mathfrak{G}$. If the class of R_α consists of g_α elements, and if $x_i = \chi_i(1)$ is the degree of χ_i, then it is well known that the number

$$\omega_i(R_\alpha) = g_\alpha X_i(R_\alpha)/x_i$$

is an algebraic integer. Introducing these quantities in (3), we obtain

$$a_{ij} = (p^d/g)x_i \sum_{\alpha=1}^{h} \omega_i(R_\alpha)\bar{\chi}_j(R_\alpha). \tag{5}$$

If χ_r is another irreducible character in B and if ω_r has the analogous significance as ω_i, then modulo a suitable prime ideal divisor $\mathfrak{p}$ of p, we have

$$\omega_i(R_\alpha) \equiv \omega_r(R_\alpha) \pmod{\mathfrak{p}}.$$

Now, (5) yields easily the congruence

$$(g/p^d)\ (a_{ij}/x_i) \equiv (g/p^d)\ (a_{rj}/x_r) \pmod{p}.$$

Let ν denote the p-adic exponential valuation, $\nu(p) = 1$. By the definition of the defect d of a block B, we can set

$$\nu(x_i) = \nu(g) - d + \lambda_i \tag{6}$$

with $\lambda_i \geq 0$ for $1 \leq i \leq m$. We shall term the integer λ_i the height of the character χ_i in B. There exist characters of height 0 in B. We choose χ_r as such a character, $\lambda_r = 0$.

If congruences are taken as congruences in the ring of local integers for p, our result becomes

$$a_{ij} \equiv (x_i/x_r)a_{rj} \pmod{p^{1+\lambda_i}}.$$

Using the symmetry of a_{ij}, we find

$$a_{ij} \equiv (x_i x_j/x_r^2)a_{rr} \pmod{p^{1+\lambda_i}}. \tag{7}$$

It follows that a_{rr} is not divisible by p, since otherwise all a_{ij} would be divisible by p, and we have already seen that this is not true. Taking $j = r$ in (7), we obtain

$$\nu(a_{ir}) = \lambda_i. \tag{8}$$

In particular, $a_{ir} \neq 0$ for $1 \leq i \leq m$.

It follows from (4) together with $D'D = C$ that

$$A^2 = p^d A. \tag{9}$$

Hence

$$\sum_{i=1}^{m} a_{ir}^2 = p^d a_{rr}. \tag{10}$$

Since $a_{ir} \neq 0$, this yields

$$(m - 1) + a_{rr}^2 \leq p^d a_{rr}.$$

The maximum of $p^d x - x^2$ is obtained for $x = p^d/2$ and hence $m - 1 \leq p^{2d}/4$. This completes the proof of Theorem 1.

The formulas developed here can be used to obtain some further results. It follows from (3) in conjunction with the orthogonality relations for group characters that $0 < a_{ii} \leq p^d$. By (9),

$$\sum_{i=1}^{m} a_{ji}^2 = p^d a_{ii} \tag{11}$$

for $i = 1, 2, \ldots, m$. If $a_{ii} = p^d$, all a_{ji} with $j \neq i$ vanish. Then (8) implies that $i = r$. Since $\nu(a_{rr}) = 0$, it follows that $d = 0$. Thus $a_{ii} < p^d$ for $d > 0$. This can also be seen easily directly.

We defined the height λ_i of a character χ_i of B by the equation (6). If $\lambda_i > 0$, then (7) shows that a_{ii} is divisible by $p^{\lambda_i + 1}$. Hence $\lambda_i + 1 < d$. We thus have a new proof of the following result.[3]

THEOREM 2. *If B is a block of defect $d \geq 2$, the height λ_i of a character χ_i in B is at most $d - 2$. For $d = 0$, 1, and 2, we have $\lambda_i = 0$ for all χ_i in B.*

There exist examples of blocks of arbitrary defect $d \geq 2$ which contain characters of height $d - 2$.

Let m_λ denote the number of characters χ_i of B of height λ;

$$m = \sum_{\lambda} m_\lambda. \tag{12}$$

Then as shown by (8), m_λ terms a_{ir} in (10) contain p with the exact exponent p^λ. Hence the method used above yields

$$(m_0 - 1) + m_1 p^2 + m_2 p^4 + \ldots \leq {}^1/_4\, p^{2d}. \tag{13}$$

In particular

$$m_\lambda < {}^1/_4 p^{2d - 2\lambda} \tag{14}$$

for $\lambda > 0$, as it is easy to see that $m_0 > 1$.

On the other hand, if B contains characters χ_i of positive height $\lambda_i = \lambda > 0$, then m_0 terms a_{ji} in (11) contain p with the exact exponent λ. Hence

$$m_0 \leq {}^1/_4 p^{2d - 2\lambda}. \tag{15}$$

On combining (12), (13), and (15) we obtain

THEOREM 3. *If the p-block B of defect d contains irreducible characters χ_i of positive height, the number m of irreducible characters in B is less than ${}^1/_2 p^{2d-2}$.*

So far we have only used p-regular elements R of $\mathfrak{G}$. If P now is an element of $\mathfrak{G}$ whose order is a power $p^a \geq 1$ of p, and if R is a p-regular element of the centralizer $\mathfrak{C}(P)$ of P, the formula (2) can be generalized to

$$\chi_i(PR) = \sum_j d_{ij}^P \varphi_j^P(R),$$

where the φ_j^P are the modular irreducible characters of $\mathfrak{C}(P)$ and where the d_{ij}^P are algebraic integers of the field Ω_a of the p^ath roots of unity. If we consider only χ_i belonging to a fixed p-block B of $\mathfrak{G}$, only characters φ_j^P have to be taken which belong to a well-determined set of p-blocks $\bar{B}$ of $\mathfrak{C}(P)$. We set

$$a_{ij}^P = (p^d/n(P)) \sum_R \chi_i(PR)\bar{\chi}_j(PR),$$

where $n(P)$ is the order of $\mathfrak{C}(P)$ and where R ranges over the p-regular elements of $\mathfrak{C}(P)$. For $P = 1$, we have $a_{ij}^P = a_{ij}$. Let A^P be the matrix of the a_{ij}^P, D^P the matrix of the d_{ij}^P, and $\mathfrak{C}^P$ the direct sum of the matrices $\bar{C}$ of the Cartan invariants of the blocks $\bar{B}$ of $\mathfrak{C}(P)$ associated with B. Then (4) can be generalized to

$$A^P = p^d D^P (C^P)^{-1} (\bar{D}^P)'.$$

It follows that the a_{ij}^P are algebraic integers of the field Ω_a. Similarly to (9), we have $(A^P)^2 = p^d A^P$. There is also a form in which (7) can be generalized. If P ranges over a system of representatives for the classes of conjugate elements whose order is a power of p, we have

$$\sum_P a_{ij}^P = p^d \delta_{ij}.$$

Finally, there exists a simple connection between the p-blocks of $\mathfrak{C}(P)$ and those of $\mathfrak{C}(P)/\{P\}$.

We now state a number of results without proof which can be shown by means of these formulas. If we use known results[4] on blocks of defect 0 and 1 and Theorem 3, we obtain the following improvement of Theorem 1.

THEOREM 1*. *If B, p, d, m have the same significance as in Theorem 1, then $m \leq p^d$ for $d = 0, 1, 2$ and $m < p^{2d-2}$ for $d > 2$.*

[4] Probably, the inequality $m \leq p^d$ holds for all d, but we have not been able to prove this. However, for certain types of defect groups $\mathfrak{D}$ of the block B, our results can be improved. For instance, we can prove:

THEOREM 4. *If the defect group $\mathfrak{D}$ of the block B in Theorem 1 is cyclic, then $m \leq p^d$.*

Actually, there is a connection between the structure of the defect group $\mathfrak{D}$ of the block B and the maximal height λ of characters in B.

THEOREM 5. *Let $\mathfrak{D}$ of order p^d be the defect group of the p-block B of $\mathfrak{G}$ and assume that there exist u elements in the center of $\mathfrak{D}$ such that no two of them are conjugate in $\mathfrak{G}$. If B contains characters χ of height $\lambda > 0$, then*

$$u < p^{d-\lambda}. \tag{16}$$

If $p = 2$, then (16) can be replaced by

$$u \leq 2^{d-\lambda-1}. \tag{16*}$$

COROLLARY. *If B is a block of full defect $d = \nu(g)$, i.e., if the defect group $\mathfrak{D}$ is a*

p-Sylow group of $\mathfrak{G}$ and if the center of $\mathfrak{D}$ contains an element P of order p^α, then B can contain characters of height λ only if

$$\lambda \leq d - \alpha.$$

For $p = 2$ and $\lambda > 0$, we even have

$$\lambda \leq d - \alpha - 1.$$

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[3] Brauer, R., *Ann. Math.*, **42**, 936–958 (1941).

[4] Cf. the paper quoted in reference 3.

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*ESTIMATES AND EXISTENCE THEOREMS FOR ELLIPTIC BOUNDARY VALUE PROBLEMS**

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Let A be a linear elliptic differential operator of order $2m$ ($m \geq 1$) with complex-valued coefficients defined on an open subset G of Euclidean n-space E^n. It is our purpose in the present note to discuss the existence and regularity of solutions of a general family of boundary-value problems of local type for A on G. An important aspect of our discussion is that it assumes no regularity conditions on the coefficients of A except continuity, makes no semiboundedness assumption on the boundary-value problem, and yields information on the nature of A realized as an operator in $L^p(G)$ under null boundary conditions for unbounded as well as bounded domains G. The key tool of our discussion is a family of *a priori* estimates for the derivatives of solutions in $L^p(G)$ (as well as in Hölder norm, provided the coefficients are Hölder continuous). Since the existence results are obtained under extremely weak assumptions upon the regularity of the coefficients of A, they are a useful step toward the construction of a theory of linear equations sufficient for the attack upon boundary value problems for nonlinear elliptic equations. The results which are discussed below are immediately extendable to the general class of elliptic systems under analogous hypotheses. In a note to follow, we shall discuss the stronger conclusions that may be obtained under similar hypotheses for the Dirichlet problem for strongly elliptic equations and systems (as well as other semi-bounded problems) and derive from these, existence theorems for mixed initial-boundary value problems for temporally inhomogeneous equations of propagation.

We shall use the notation $D_j = \mathrm{i}^{-1}\,\partial/\partial x_j$, while for an n-tuple α of nonnegative $(\alpha_1, \ldots, \alpha_n)$, we set $D^\alpha = {D_1}^{\alpha_1} \ldots {D_n}^{\alpha_n}$, $|\alpha| = \sum_j \alpha_j$. Similarly, for a complex n-vector $\zeta = (\zeta_1, \ldots, \zeta_n)$, $\zeta^\alpha = {\zeta_1}^{\alpha_1} \ldots {\zeta_n}^{\alpha_n}$. In terms of this notation, the differential operator A may be written as $\sum_{|\alpha| \leq 2m} a_\alpha(x) D^\alpha$, and its characteristic form is, $a(x, \zeta) = \sum_{|\alpha| = 2m} a_\alpha(x)\zeta^\alpha$. If $B_1, \ldots, B_m$ are m differential operators of order $<2m$ defined on a neighborhood of the boundary of G, each B_j may be written as

$\sum_{|\beta|<2m} b_{j,\beta}(x)\, D^\beta$ and its characteristic form as $b_j(x, \zeta) = \sum_{|\beta|=r_j} b_{j,\beta}(x)\zeta^\beta$, where r_j is the order of B_j.

The domain G with boundary Γ is said to be uniformly regular of class $C^s (s \geq 1)$ if there exists an integer $r > 0$ and sequences $\{N_j\}$ of open subsets of E and $\{\phi_j\}$ of homeomorphisms of N_j on the unit ball in E^n such that:

(1) Any $(r + 1)$ distinct sets N_j have an empty intersection;
(2) $\phi_j(N_j \cap G) = \{y: |y| < 1, y_n > 0\}$, $\phi_j(N_j \cap \Gamma) = \{y: |y| < 1, y_n = 0\}$;
(3) If $N_j' = \phi_j^{-1}(|y| < 1/2)$, $\bigcup_j N_j'$ contains the $(1/r)$-neighborhood of Γ;
(4) If ϕ_{jk} and ψ_{jk} are the kth coordinates of ϕ_j and ϕ_j^{-1}, respectively, then for x in N_j, y in $\phi_j(N_j)$,

$$|(D^\alpha \phi_{jk})(x)| \leq r, \quad |(D^\alpha \psi_{jk})(y)| \leq r, \text{ for } |\alpha| \leq s.$$

We shall assume that G is uniformly regular of class C^{2m} and consider boundary value problems for $Au = f$.

For $j \geq 0$, $1 < p < \infty$, we introduce the Banach space $W^{j,p}(G) = \{f: D^\alpha f \in L^p(G)$ for $|\alpha| \leq j$, derivatives taken in the distribution sense$\}$, with the norm $\|f\|_{j,p} = \{\sum_{|\alpha|\leq j} \|D^\alpha f\|_{L^p(G)}^p\}^{1/p}$. Under our assumption on G, the family $C_c^\infty(E^n)$ of infinitely differentiable functions with compact support in E^n forms a dense subset of $W^{2m,p}(G)$ for every $p > 1$ if each function is restricted to G. If we use normalized tangential derivatives on Γ and integrate with respect to the $(n - 1)$-dimensional area element induced on Γ by E^n, we may similarly define the spaces $W^{j,p}(\Gamma)$. For each β with $|\beta| < 2m$, we define a linear mapping π_β of $C_c^\infty(E^n)$ into $W^{2m-1-|\beta|,p}(\Gamma)$, by $\pi_\beta(f) = D^\beta f|_\Gamma$.

If G is uniformly regular of class C^{2m}, the mapping π_β is a bounded linear mapping from the dense subset $C_c^\infty(E^n)$ of $W^{2m,p}(G)$ into $W^{2m-1-|\beta|,p}(\Gamma)$, and hence may be extended by continuity to a mapping defined on all of $W^{2m,p}(G)$. If we compose this extended mapping π_β with the injection mapping of $W^{2m-1-|\beta|,p}(\Gamma)$ into $L^p(\Gamma)$, we obtain a continuous mapping of $W^{2m,p}(G)$ into $L^p(\Gamma)$ which we shall denote by $D^\beta|_\Gamma$.

Similarly if, for j a nonnegative integer, $0 < h < 1$, we define the Banach space $C^{j,h}(G)$ as $\{f: f \in C^j(\bar{G}), H_\alpha(f) = \sup_{x,y\in G} |(D^\alpha f)(x) - (D^\alpha f)(y)| \cdot |x - y|^{-h} < \infty$ for $|\alpha| = j\}$, with the norm $\|f\|_{C^{j,h}(G)} = \sum_{|\alpha|\leq j} \sup_{x\in G} |D^\alpha f(x)| + \sum_{|\alpha|=j} H_\alpha(f)$, then π_β may be defined for each β with $|\beta| \leq 2m - 1$ as the restriction of $D^\beta f$ to Γ. In this case π_β is a bounded linear mapping of $C^{2m,h}(G)$ into $C^{2m-|\beta|,h}(\Gamma)$, where the latter is defined analogously using the normalized tangential derivatives on Γ, and $D^\beta|_\Gamma$ is the corresponding mapping of $C^{2m,h}(G)$ into $C^{0,h}(\Gamma)$.

Suppose that we are given m partial differential operators $B_1, \ldots, B_m$ with coefficients $b_{j,\beta}(x)$ uniformly continuous and bounded on the $(1/r)$-neighborhood of Γ. We may define corresponding mappings $B_1, \ldots, B_m$ of $W^{2m,p}(G)$ into $L^p(\Gamma)$ and of $C^{2m,h}(G)$ into $C^{0,h}(\Gamma)$, by $B_j f = \sum_{|\beta|<2m} b_{j,\beta}(x)\, D^\beta f|_\Gamma$. Each of the mappings B_j is continuous in the norm topologies of the domain and range spaces. More generally, we may localize the definition of the operators B_j by considering B_j as acting on functions f which lie in $W^{2m,p}(N)$ for some neighborhood N of each point of the boundary Γ.

Our basic *a priori* estimates are the following:

$(W)_p$ Suppose that u is a function lying in $W^{2m,p_1}(N)$ for a neighborhood N in

G of each point of the closure of G, $(p_1 > 1)$, and satisfies the conditions $B_j u = 0$, $1 \leq j \leq m$, $Au \in L^p(G)$, $(1 < p < \infty)$. Then u lies in $W^{2m,\,p}(G)$ and there exists a constant c_p, independent of p_1 or u, such that

$$\|u\|_{2m,\,p} \leq c_p\{\|Au\|_{L^p(G)} + \|u\|_{0,\,p}\}.$$

$(C)_h$ If in addition, $Au \in C^{0,\,h}(G)$ for some h with $0 < h < 1$, then u lies in $C^{2m,\,h}(G)$. There exists a constant c_h, independent of u or p_1, such that

$$\|u\|_{C^{2m,\,h}(G)} \leq c_h\{\|Au\|_{C^{0,\,h}(G)} + \|u\|_{C^{0,\,h}(G)}\}.$$

Let us formulate the precise hypotheses under which these estimates hold. We shall assume that A is uniformly elliptic on G, i.e., there exists a constant $c_1 > 0$ such that $|a(x, \xi)| \geq c_1$ for x in G and all real n-vectors ξ with $|\xi| = 1$. At a boundary point x of G, let n be a unit normal vector to Γ and let t denote any unit tangent vector to Γ.

(E_1) We assume that the polynomial $a(x, t + \lambda n)$ in the complex variable λ has exactly m roots with positive imaginary part (as is always the case for $n > 2$), and that if $p_{j,\,k}(x, t) = \int_{C_t} \lambda^{j-1} b_k(x, t + \lambda n)\{a(x, t + \lambda n)\}^{-1}\, d\lambda$, where C_t is a curve in the upper λ half-plane surrounding the zeroes of $a(x, t + \lambda n)$, $(1 \leq j \leq m, 1 \leq k \leq m)$, then there exists $c_2 > 0$ such that $|Det(p_{jk}(x, t))| \geq c_2$ for all tangential vectors t to Γ at x with $|t| = 1$, and all x in Γ.

THEOREM 1.[1] *Suppose that the coefficients a_α of A are bounded measurable on G with a_α uniformly continuous on G for $|\alpha| = 2m$. Suppose further that G is uniformly regular of class C^{2m}, the coefficients $b_{j,\,\beta}$ of B_j are uniformly bounded and continuous in the (r^{-1})-neighborhood of Γ, that A is uniformly elliptic on G, and that E_1 holds. Then the a priori estimate $(W)_p$ is valid for all p with $1 < p < \infty$.*

THEOREM 2. *Suppose that A and the B_j satisfy the conditions of Theorem 1, with the additional hypothesis that the coefficients a_α and $b_{j,\,\beta}$ satisfy uniform Hölder conditions with exponent h on G. Suppose also that G is regular of class C^{2m+1}. Then the a priori estimate $(C)_h$ is valid.*

The proofs of Theorems 1 and 2 which are lengthy and technical will appear elsewhere. We remark that the condition (E_1) for ellipticity of the boundary value problem is essentially due to Lopatinski.[2] It is essentially equivalent to the uniform form of the following condition which has been considered by a number of recent writers:[3]

(E_2) Let x be a boundary point, t a unit tangent vector to Γ at x, n a unit normal vector to Γ at x. Let $q(x, t, \lambda)$ be the product of all linear factors of $a(x, t + \lambda n)$ with roots having positive imaginary parts, where $a(x, t + \lambda n)$ is considered as a polynomial in the complex variable λ. Then the polynomials in λ, $b_1(x, t + \lambda n)$, $\ldots$, $b_m(x, t + \lambda n)$ form a linearly independent set modulo $q(x, t, \lambda)$ for every t.

LEMMA 1. *(E_2) holds if and only if $|Det(p_{jk}(x, t))| \geq c_2(x) > 0$ for all tangent vectors t to Γ at x with $|t| = 1$.*

Proof of Lemma 2: If the $b_j(x, t + \lambda n)$ are linearly dependent modulo $q(x, t, \lambda)$ for some unit tangent vector t to Γ at x i.e., $\sum_k c_k b_k(x, t + \lambda n) = r(\lambda) q(x, t, \lambda)$ for some polynomial $r(\lambda)$, $(c_k \not\equiv 0)$ it follows that

$$\sum_k c_k p_{jk}(x, t) = \int_{C_t} \lambda^{j-1} (\sum_k c_k b_k(x, t + \lambda n))\, a(x, t + \lambda n)^{-1}\, d\lambda =$$
$$\int_{C_t} \lambda^{j-1} r(\lambda) q(x, t, \lambda) a(x, t + \lambda n)^{-1}\, d\lambda = 0$$

for every j since the integrand is analytic inside C_t.

To establish the converse, it suffices by continuity to consider the case where $Det(p_{jk}(x, t)) = 0$. Then there exists a non-null sequence $\{c_k\}$ such that $\sum_k c_k p_{jk}(x, t) = 0$ for all j. If we set $Q(\lambda) = \sum_k c_k b_k(x, t + \lambda n)$, we have $\int_{C_t} \lambda^{j-1} Q(\lambda) a(x, t + \lambda n)^{-1}\, d\lambda = 0$ for $1 \leq j \leq m$. If $h(\lambda)$ is the greatest common divisor of $Q(\lambda)$ and $q(x, t, \lambda)$, either $h(\lambda)$ is of lower degree than $q(x, t, \lambda)$ or $q(x, t, \lambda)$ divides $Q(\lambda)$. In the second case (E_2) does not hold. To complete the argument, it suffices to show that the first case is impossible. For that case, there exist polynomials $s(\lambda)$ and $s_1(\lambda)$ such that $s(\lambda)Q(\lambda) + s_1(\lambda)q(x, t, \lambda) = h(\lambda)$. Multiplying by the polynomial $\lambda^r \cdot a(x, t + \lambda n) \cdot \{q(x, t, \lambda)\}^{-1}$, where $r = (m - 1) - \text{order}(h)$, we obtain polynomials $S(\lambda)$ and $S_1(\lambda)$ such that $S(\lambda)Q(\lambda) + S_1(\lambda)\, q(x, t, \lambda) = \lambda^r h(\lambda)\, a(x, t + \lambda n)\, \{q(x, t, \lambda)\}^{-1}$. Multiplying by $\{a(x, t + \lambda n)\}^{-1}$ and integrating around C_t, we obtain $0 = \int_{C_t} \lambda^r h(\lambda)\, \{q(x, t, \lambda)\}^{-1}\, d\lambda$. But the integrand has a zero of order one at ∞ and poles only inside C_t, so that the integral is nonzero, yielding a contradiction.

The remainder of our discussion is concerned with deducing consequences of Theorem 1. Analogous arguments may be given in the spaces $C^{2m,\,p}(G)$ on the basis of Theorem 2, but we shall not consider them explicitly here.

THEOREM 3. *Suppose that* $(W)_p$ *holds for the boundary value problem* $(A, B_1, \ldots, B_m)$. *Consider* A *as an operator with dense domain* $D(A) = \{u: u \in W^{2m,\,p}(G), B_j u = 0 \text{ for } 1 \leq j \leq m\}$ *in* $L^p(G)$ *and range in* $L^p(G)$. *Then:*

(*a*) A *is a closed operator in* $L^p(G)$. *If in addition,* G *is a bounded domain, then:*

(*b*) *The null-space of* A *is finite dimensional.*

(*c*) $R(A)$, *the range of* A, *is a closed subspace of* $L^p(G)$.

Proof of (*a*): Let u_k be an infinite sequence from $D(A)$ with $u_k \to u_0$, $Au_k \to v_0$ in $L^p(G)$. By $(W)_p$, $\{u_k\}$ is a Cauchy sequence in $W^{2m,\,p}(G)$ and converges to an element of $W^{2m,\,p}(G)$ which must coincide with u_0. Since $B_j u_k = 0$ for every k and since B_j is a continuous mapping of $W^{2m,\,p}(G)$ into $L^p(G)$, $B_j u_0 = 0$. Thus $u_0 \in D(A)$. But A is a continuous mapping of $W^{2m,\,p}(G)$ into $L^p(G)$, so that $u_k \to u_0$ in $W^{2m,\,p}(G)$ implies that $Au_k \to Au_0$ in $L^p(G)$, i.e., $v_0 = Au_0$ and A is a closed operator.

Proof of (*b*): For u in $N(A)$, the null-space of A, we have by $(W)_p$, $\|u\|_{2m,\,p} \leq c_p \|u\|_{0,\,p}$. However, if G is bounded and uniformly regular, every bounded set in $W^{2m,\,p}(G)$ is pre-compact in $L^p(G)$. Thus the unit ball in $N(A)$, considered as a closed subspace of $L^p(G)$, is pre-compact, and $N(A)$ must be finite-dimensional.

Proof of (*c*): Since $N(A)$ is finite dimensional, there exists a bounded projection P of $L^p(G)$ on $N(A)$. If $H = (I - P)\,(L^p(G))$, $D(A) = N + \{D(A) \cap H\}$. Thus A is an injective mapping of $D(A) \cap H$ onto $R(A)$, and H is closed. Since A is a closed operator by (*b*) and A is one-to-one on $D(A) \cap H$, to show that $R(A)$ is closed is equivalent to showing that there exists a constant $c_3 > 0$ such that $\|Au\|_{0,\,p} \geq c_3 \|u\|_{0,\,p}$ for all u in $D(A) \cap H$. Suppose the contrary. Then there must exist a sequence $\{u_k\}$ from $D(A) \cap H$ such that $\|u_k\|_{0,\,p} = 1$, while $Au_k \to 0$ in $L^p(G)$. By $(W)_p$, the sequence $\{u_k\}$ is bounded in $W^{2m,\,p}(G)$, and this latter space being separable and reflexive (since it is uniformly convex) there exists a subsequence, which we may identify with the original sequence, converging weakly to a function u_0 in $W^{2m,\,p}(G)$. Weak convergence in $W^{2m,\,p}(G)$ together with the compactness of the imbedding mapping of $W^{2m,\,p}(G)$ in $L^p(G)$, however, imply

that $u_k \to u_0$ strongly in $L^p(G)$ and hence that $\|u_0\|_{0,p} = 1$. Since H is closed in $L^p(G)$, u_0 must lie in H. Since $B_j u_k = 0$ for each j and k, and since B_j is a weakly continuous map of $W^{2m,p}(G)$ into $L^p(\Gamma)$, $B_j u_0 = 0$, i.e., $u_0 \in D(A)$. Since A is a continuous and hence weakly continuous mapping of $W^{2m,p}(G)$ into $L^p(G)$, Au_k converges weakly to Au_0 in $L^p(G)$. Since $Au_k \to 0$ strongly in $L^p(G)$, $Au_0 = 0$. Thus u_0 is a non-null element of $N(A) \cap \{D(A) \cap H\}$, which is a contradiction.

THEOREM 4. *Suppose that the boundary value problem $(A, B_1, \ldots, B_m)$ and the domain G satisfy the hypotheses of Theorem 1, and that in addition G is bounded. Then the codimension of $R(A)$ in $L^p(G)$ is finite.*

Proof of Theorem 4: We sketch the basic idea of the proof for the Dirichlet problem. For the case of more general boundary operators $\{B_j\}$, the localization and the treatment of the variable boundary terms involve more precise estimates of the type $(W)_p$ for solutions of $Au = f$ with inhomogeneous boundary conditions.

We may find a finite open covering $\{N_j\}$ of the closure of G of the type described in the definition of uniform regularity such that (1) for each N_j not intersecting Γ, there exists a bounded linear mapping T_j of $L^p(N_j)$ such that $T_j u$ is locally in $W^{2m,p}$ in the interior of N_j while $AT_j u = u$; (2) For each N_j which intersects Γ, there exists a bounded linear mapping T_j of $L^p(N_j \cap G)$ such that $T_j u$ lies in $W^{2m,p}$ on a neighborhood of each point of $N_j \cap cl(G)$, $AT_j u =$ in $N_j \cap G$, while $D^\beta T_j u = 0$ on $N_j \cap \Gamma$ for $|\beta| < m$. Let $\{\phi_j\}$ be an infinitely differentiable partition of unity corresponding to the covering $\{N_j\}$. For each u in $L^p(G)$, let $P_j u$ be the restriction of u to $N_j \cap G$. Finally set $Uu = \sum_j \phi_j T_j(P_j u)$.

U is obviously a bounded linear transformation of $L^p(G)$. By the construction of the T_j for the sets N_j intersecting Γ, Uu satisfies the null Dirichlet conditions locally at each point of the boundary. Since $T_j(P_j u)$ is in $W^{2m,p}$ on a neighborhood of every point of $N_j \cap cl(G)$ and since the support of ϕ_j in G is a pre-compact subset of that set, Uu lies in $W^{2m,p}(G)$. Finally

$$A(Uu) = \sum_j \phi_j A T_j(P_j u) + \sum_{|\beta| \leq 2m-1} \psi_{j,\beta} D^\beta T_j(P_j u) = u + Su,$$

where $Su = \sum_{j,\beta} \psi_{j,\beta} D^\beta T_j(P_j u)$.

Since T_j maps $L^p(N_j \cap G)$ into functions locally in $W^{2m,p}$, by the closed graph theorem, $D^\beta T_j(P_j u)$ is a bounded mapping into $W^{1,p}(K)$ for any compact subdomain K of $N_j \cap (cl(G))$ and hence a compact mapping into $L^p(K)$ if K is smoothly bounded. Since the support of $\psi_{j,\beta}$ is such a domain, S is a compact linear transformation of $L^p(G)$. But $R(A)$ contains $R(AU) = R(I + S)$, and by the Riesz theory of compact operators, $R(A)$ has finite co-dimension.

Since A is a closed operator in $L^p(G)$, it has a densely defined adjoint operator A^* in $L^q(G)$, where $q = p/(p-1)$. If G is bounded, the range of A is closed by Theorem 3, and therefore so is the range of A^*. Further, $R(A)$ is the orthogonal complement in the obvious sense of $N(A^*)$, and by Theorem 4, $N(A^*)$ is finite dimensional.

The remainder of our discussion is devoted to giving a more concrete characterization of A^* under more restrictive hypotheses on A, namely the continuity of the coefficients of its formal adjoint. The adjoint differential operator A' to A may be defined by $A'u = \sum_{|\alpha| \leq 2m} D^\alpha(\bar{a}_\alpha(x)u)$, with differentiation in the sense of the theory of distributions, and may be written in the form $\sum_{|\alpha| \leq 2m} a_\alpha'(x) D^\alpha$, where the a_α' are distributions on G. We shall assume henceforward that A' satisfies the same

regularity conditions as the original differential operator A. We suppose also that we are given a family of m differential operators $(B_1', \ldots, B_m')$ of the same type as the family $\{B_j\}$. The family $\{B_j'\}$ is said to be formally adjoint to the family $\{B_j\}$ with respect to A if the following condition is satisfied:

A function v in $W^{2m,\,q}(G)$ satisfies the equation $(Au, v) = (u, A'v)$ for all u in $W^{2m,\,p}(G)$ such that $B_ju = 0$, $1 \leq j \leq m$, if and only if $B_j'v = 0$ for $1 \leq j \leq m$.

The Dirichlet boundary conditions are self-adjoint in the above sense for every elliptic differential operator A.

The pair of boundary value problems $(A, B_1, \ldots, B_m)$ and $(A', B_1', \ldots, B_m)$ are said to be regularizable if there exist a pair of sequences $(A_k, B_{1,k}, \ldots, B_m)$ and $(A_k', B_1', \ldots, B_{m,k}')$, where A_k, for each k, is an elliptic differential operator, A_k' is its adjoint, the two boundary problems are formally adjoint for each k, the coefficients of A_k, A_k', $B_{j,k}$, and $B_{j,k}'$ are locally of class C^{2m} in the neighborhood of each point of $cl(G)$, while each of the coefficients converges uniformly on G as $k \to \infty$ to the corresponding coefficient of A, A', B_j, or B_j'. The Dirichlet problem for an arbitrary elliptic operator A is regularizable, since we may keep the boundary conditions fixed and take the convolution of all the coefficients of A and A' with a sequence of functions in $C_c^\infty(E^n)$ converging to the Dirac δ. Any pair of formally adjoint problems with coefficients of class C^{2m} are obviously regularizable.

THEOREM 5. *Let A be an elliptic differential operator on G, A' its formal adjoint. Suppose that the pair of boundary value problems $(A, B_1, \ldots, B_m)$ and $(A', B_1', \ldots, B_m')$ are formally adjoint and regularizable, and suppose that each of the two problems satisfies the conditions of Theorem 1. Suppose also that G is locally of class C^{4m} as well as being uniformly regular of class C^{2m}. Let A and A' be defined with $D(A) = \{u: u \in W^{2m,\,p}(G), B_ju = 0 \text{ for } 1 \leq j \leq m\}$, $D(A') = \{u: u \in W^{2m,\,q}(G), B_j'u = 0 \text{ for } 1 \leq j \leq m\}$, $p^{-1} + q^{-1} = 1$. Then if A is considered as an operator in $L^p(G)$, A' in $L^q(G)$, we have $A^* = A'$, $(A')^* = A$.*[4]

COROLLARY TO THEOREM 5. *If we suppose also that G is bounded, then $Au = f$ has a solution u in $D(A)$ for a given f if and only if $(f, v) = 0$ for all solutions v in $D(A')$ of the equation $A'v = 0$. The family of such v, $N(A')$, is of finite dimension.*[5]

Proof of Theorem 5: Because of $(W)_p$ holding for (A', B_j'), it suffices to show that if v lies in $L^q(G)$ and satisfies the equation $(Au, v) = (u, f)$ for an f in $L^q(G)$ and all u in $D(A)$, then v is locally in $W^{2m,\,p_1}$ for every point of the closure of G for some $p_1 > 1$. Further if $A^* = A'$, it follows from the reflexivity of $L^p(G)$, since A is a closed operator, that $(A')^* = ((A)^*)^* = A$. Since the pair of problems is interchangeable, we may assume that $p \leq 2$, $q \geq 2$. It follows for bounded domains that the theorem for general p follows from the result for $p = 2$. Indeed on a bounded domain G, the domain of A in $L^2(G)$ lies in the corresponding domain in $L^p(G)$ for $p < 2$, while every f in $L^q(G)$ also lies in $L^2(G)$. A similar but more involved argument based on localization gives the same result for unbounded domains. Thus we need to consider only the case $p = 2$.

Next, we observe that we may restrict our consideration to problems of the type of $(A_k, \{B_{j,k}\})$ and $(A_k', \{B_{jk}'\})$ where the coefficients are locally of class C^{2m}. Indeed, suppose that $(A_k)^* = A_k'$ for each of the approximating regularized problems. Then for each k which is sufficiently large, $(W)_2$ is valid for A_k and A_k' with a constant c_2 independent of k. For each such k, $A_k'A_k + I$ which is self-adjoint

in $L^2(G)$ and $\geq I$, has an inverse R_k defined on all of $L^2(G)$ with $\|R_k\| \leq 1$. For a given f in $L^2(G)$, let $u_k = R_k f$, $w_k = A_k u_k$. By its definition, w_k lies in $D(A_k')$ while $A_k' w_k = -u_k + f$. Further, $\|u_k\| \leq \|f\|$, and $\|w_k\|^2 = \|A_k u_k\|^2 = (f, u_k) - \|u\|^2 \leq \|f\|^2$. It follows from $(W)_2$ that $\|u_k\|_{2m,2} + \|w_k\|_{2m,2} \leq 2c_2 \|f\|$ for all k. By choosing a subsequence, we may assume that $u_k \to u_0$, $w_k \to w_0$ weakly in $W^{2m,2}(G)$. But weak convergence of u_k and w_k in $W^{2m,2}(G)$ implies weak convergence of $D^\beta u_k$ and $D^\beta w_k$ in $L^2(\Gamma)$ for $|\beta| \leq 2m - 1$, and indeed local strong convergence. It follows that $u_0 \in D(A)$, $w_0 \in D(A_0')$, since the coefficients of $B_{j,k}$ and $B_{j,k}'$ converge uniformly to those of B_j and B_j'. Moreover, A_k and A_k' considered as mappings with domain $W^{2m,2}(G)$ and range in $L^2(G)$ are convergent in norm to A and A' respectively as $k \to \infty$. It follows that $f = A_k' w_k$ converges weakly in $L^2(G)$ to $A' w_0$, i.e., $A' w_0 = f$, and that $A_k u_k = w_k$ converges weakly in $L^2(G)$ to $A u_0$. Thus u_0 lies in $D(AA')$ and $AA' u + u = f$. Thus $AA' + I$, which is contained in $(A')^* A' + I$, must be equal to the latter, since both are onto and the bigger has a bounded inverse. It follows that $AA' = (A')^* A'$, and similarly, $A'A = A^* A$. In particular, A' coincides with A^* on $R(A)$. But, since $AA' + I$ is onto, every v in $L^2(G)$ may be written in form $AA' u + u$, where $AA' u$ lies in $R(A)$ and u lies in $D(A')$. If $v \in D(A^*)$, $v - u \in D(A^*) \cap R(A) \subset D(A')$, and $v = u + (v - u) \in D(A')$. Therefore, $A^* = A'$, and the conclusion of the theorem follows for A.

To complete the proof, we assume that A, A', B_j, and B_j' all have coefficients locally of class C^m. Suppose u lies in $D(A^* A)$. Then for all v in $D(A)$, $(Au, Av) = (f, v)$ where $f = A^* Av \in L^2(G)$. It follows from $(W)_2$ that u is a solution of the variational boundary value problem associated with the coercive form $[u, v] = (Au, Av)$ on the subspace $D(A)$ of $W^{2m,2}(G)$. Since all the coefficients are locally of class C^{2m}, it follows from the regularity theory of solutions of coercive variational boundary value problems that u is locally of class $W^{4m,2}$ in the neighborhood of every point of the closure of G.[6] Thus if $w = Au$, w is locally of class $W^{2m,2}$, $A^* w = A' w$, and $(w, Au) = (A' w, u)$ for all v in $D(A)$. It follows that $w \in D(A')$. Thus $Au \in D(A')$, $u \in D(A'A)$, and $A'Au = A^* A$. Similarly, $AA' = (A')^* A'$. It follows that A' and A^* coincide on $D(A^*) \cap R(A)$. But since AA' is self-adjoint and positive, $(AA' + I)$ maps onto $L^2(G)$. Hence, as in the preceding paragraph, $D(A^*) \subset \{D(A^*) \cap R(A)\} + D(A') \subset D(A')$. Therefore, $A^* = A'$ and the theorem is established.

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[1] Estimates in $W^{2m,2}$ for the Dirichlet problem for strongly elliptic operators A on bounded domains were obtained by Guseva, O. V., *Dokladi Akad. Nauk S.S.S.R.* (n.s.), **102**, 1068–1072 (1955) and Browder, F. E., *Comm. Pure App. Math.*, **9**, 351–361 (1956). (See also Nirenberg, L., *Comm. Pure App. Math.*, **8**, 648–674 (1955), where a slightly weaker result was obtained.) $W^{2m,2}$ estimates in the Dirichlet problem for the general elliptic operator were obtained by Schechter, M., *Am. J. Math.*, **79**, 431–443 (1957). More general variational boundary value problems for strongly elliptic operators have been treated by the writer (*loc. cit.*), N. Aronszajn, and K. T. Smith (unpublished), and Agmon, S., *Journal d'Analyse Math.*, **6** (1958–1959) (to appear). $W^{2m,p}$ estimates for the Dirichlet problem for strongly elliptic operators on unbounded domains have been applied by the writer [*Compt. Rend. Acad. Sci.*, **246**, 526–528, 1363–1365 (1958)] to the study of the spectral theory of these operators.

[2] Lopatinski, Ya. B., *Ukranian Mat. J.*, **5**, 123–151 (1953). The proofs of Theorems 1 and 2 are based upon constructions of integral representations of solutions using techniques developed in Lopatinski's paper. In the case of Theorem 1, we also utilize the methods and results of the Calderon-Zygmund theory of singular integral operators in L^p spaces.

[3] See Agmon (*loc. cit.*) and L. Hörmander, *Acta. Math.*, **99**, 225–264 (1958), where he refers to unpublished results of Agmon, Douglis, and Nirenberg and of Schechter. Hörmander shows for operators and boundary operators with constant coefficients on a half-space, under homogeneity conditions, that (E_2) is a n.a.s.c. for all solutions of the boundary value problem in C^{2m} to be infinitely differentiable up to the boundary.

[4] For unbounded G, Theorem 5 contains an extension of a result of the writer for the Dirichlet problem for strongly elliptic operators (see the *Compt. Rend.* notes cited in reference 3).

[5] The Corollary to Theorem 5 contains a strengthened form of the theorem of Schechter, M., *Bull. A.M.S.*, **64**, 371–372 (1958), which states essentially that for the Dirichlet problem on a bounded domain and with C^∞ coefficients, if $N(A') = \{0\}$, then $R(A) \supset L^2(G) \cap C^\infty(\bar{G})$.

[6] The conclusion follows from the results of Aronszajn, N., and K. T. Smith, which have been reproduced in the Tata Institute Lectures on Elliptic Differential Equations of J. L. Lions, Bombay, 1958.

ON THE HOMOLOGY AND HOMOTOPY DECOMPOSITION OF CONTINUOUS MAPS

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1. Introduction.—In this note we discuss two decompositions of a continuous map of 1-connected spaces which stand in a heuristic duality to each other. The homology (Moore) decomposition[1] of the map $f: X \to Y$ reduces to a homology decomposition[2] of Y when X is reduced to a point, while the homotopy (Postnikov) decomposition of f reduces to a Postnikov system for X when Y is reduced to a point.

Considerable use is made here of the notions of homology, cohomology, and homotopy groups of maps.[3, 4] By framing our definitions in terms of the map itself, we avoid resorting to replacements by mapping cylinders or equivalent fibrations, and, further, no transgression theorems are required. The resulting simplicity, together with the duality, are the features of the treatment.

2. Basic Facts.—We collect together in this section the basic results we require. We consider maps and homotopies in the category of 1-connected spaces with base points o. There is a Hurewicz homomorphism $\theta: \pi_m(f) \to H_m(f)$.

PROPOSITION 2.1. *If* $H_r(f) = 0$, $r < n$, *then* $\theta: \pi_n(f) \cong H_n(f)$.

There are coefficient theorems in homotopy[5] and cohomology; namely, for an arbitrary abelian group G,

PROPOSITION 2.2. (*a*) *The sequence*

$$0 \to Ext(G, \pi_{n+1}(f)) \xrightarrow{\tilde{\gamma}} \pi_n(G; f) \xrightarrow{\eta} Hom(G, \pi_n(f)) \to 0$$

is exact;

(*b*) *The sequence*

$$0 \to Ext(H_{n-1}(f), G) \xrightarrow{\eta} H^n(f; G) \xrightarrow{\eta} Hom(H_n(f), G) \to 0$$

is exact.

We recall the definition of η in 2.2(a). Let $\{u, v\} \in \pi_n(G; f)$, given by the map

$$\begin{array}{ccc} K'(G, n-1) & \xrightarrow{u} & X \\ \downarrow i & & \downarrow f \\ CK'(G, n-1) & \xrightarrow[v]{} & Y \end{array}$$

of i into f ($K'(f, n-1)$ denotes a space with one nonvanishing integer homology group, $CK'(G, n-1)$ its cone). Then $\theta\ \pi_n(i) \cong G$, so $\{u, v\}$ induces $\{u, v\}_{*1}$: $G \to \pi_n(f)$ and $\eta\{u, v\} = \{u, v\}_*$. We may give a dual description for η in 2.2(*b*).

There are homology and homotopy exact sequences for a triple;[4] namely, if $f = gh$, then

PROPOSITION 2.3. (*a*) *There is an exact sequence*

$$\ldots \to H_r(h) \xrightarrow{(1,g)} H_r(f) \xrightarrow{(h,1)} H_r(g) \to H_{r-1}(h) \to \ldots;$$

(*b*) *There is an exact sequence*

$$\ldots \to \pi_r(h) \xrightarrow{(1,g)_*} \pi_r(f) \xrightarrow{(h,1)_*} \pi_r(g) \to \pi_{r-1}(h) \to \ldots.$$

These are, of course, corresponding sequences for groups with coefficients and for cohomology, but we will make no use of these.

3. The Construction.—We suppose $H_r(f) = 0$, $r < n$, $H_n(f) \neq 0$. Then θ: $\pi_n(f) \cong H_n(f)$, and by 2.2(*a*) we may choose $\{u, v\} \in \pi_n(H_n(f); f)$ so that $\eta\{u, v\} = \theta^{-1}$. Thus we have a map $(u, v): i \to f$,

$$\begin{array}{ccc} K'(H_n(f), n-1) & \xrightarrow{u} & X \\ {\scriptstyle i}\downarrow & & \downarrow f \\ CK'(H_n(f), n-1) & \xrightarrow{v} & Y, \end{array} \tag{3.1}$$

and $(u, v)_*: H_n(i) \cong H_n(f)$.

Form X_1 by attaching $CK'(H_n(f), n-1)$ to X by means of u. Then (u, v) admits the canonical factorization

$$\begin{array}{ccccc} K'(H_n(f), n-1) & \xrightarrow{u} & X & \xrightarrow{1} & X \\ {\scriptstyle i}\downarrow & & \downarrow{\scriptstyle j_1} & & \downarrow f \\ CK'(H_n(f), n-1) & \xrightarrow{w} & X_1 & \xrightarrow{f_1} & Y. \end{array} \tag{3.2}$$

Here X_1 is obtained from $X \cup CK'(H_n(f), n-1)$ by identifying $u(z)$ with $i(z)$ for $z \in K'(H_n(f), n-1)$; $j_1(x) = x$, $x \in X$; $w(p) = p$, $p \in CK'(H_n(f), n-1)$; $f_1(x) = f(x)$, $f_1(p) = v(p)$.

Since i, j_1 are cofibrations and (u, w) induces the identity on the cofibers, it follows that $(u, w)_*: H_*(i) \cong H_*(j_1)$. Thus $H_r(j_1) = 0$, $r \neq n$, and

$$(1, f_1)_*: H_n(j_1) \cong H_n(f). \tag{3.3}$$

We now apply 2.3(*a*) and obtain

$$(j_1, 1)_*: H_r(f_1) \cong H_r(f), \qquad r \neq n \tag{3.4}$$

$$H_n(f_1) = 0. \tag{3.5}$$

We have thus factored f as $f_1 j_1$, where j_1 is an inclusion cofibration with cofiber $K'(H_n(f), n)$ and f_1 is obtained from f by killing $H_n(f)$.

Now suppose that $H_r(f) = 0$ except for $r = n_1, n_2, \ldots$, where $n_1 < n_2 < \ldots$. Then for each k we obtain, by iterating the construction above, a factorization

$$X \xrightarrow{j_1} X_1 \xrightarrow{j_2} X_2 \to \ldots \xrightarrow{j_k} X_k \xrightarrow{f_k} Y, \qquad f = f_k j_k \ldots j_2 j_1, \tag{3.6}$$

where the cofiber of the inclusion j_r is $K'(H_{n_r}(f), n_r)$, $r = 1, \ldots, k$, and $H_r(f_k) = 0$, $r < n_{k+1}$, $H_r(f_k) \cong H_r(f)$, $r \geq n_{k+1}$.

If the sequence $\{n_i\}$ terminates with $k = m$, say, then $H_r(f_m) = 0$ for all r, so that f_m is a singular homotopy equivalence. We then describe $f = f_m j_m \ldots j_2 j_1$ as a *homology decomposition* of f. We observe, and emphasize, that if X, Y are finite-dimensional polyhedra, then the sequence $\{n_i\}$ terminates; moreover in this case the factors j_i may be chosen to be polyhedral inclusions so that f_m will be a homotopy equivalence. If the sequence $\{n_i\}$ does not terminate we may form $X_\infty = \cup X_k$, giving it the weak topology, and define $f_\infty: X_\infty \to Y$ by $f_\infty \mid X_k = f_k$. Then f_∞ is a singular homotopy equivalence. We would then write, formally,

$$f = f_\infty \ldots j_k \ldots j_2 j_1 \tag{3.7}$$

and call (3.7)—or the set of maps $(j_k, f_k, k = 1, 2, \ldots)$—a *homology decomposition*[1] of f. The k'-*classes* of the decomposition are the classes $\{u_k\} \in \pi_{n_k-1}(H_{n_k}(f);\ X_{k-1})$, where $\{u_k, v_k\}$ was chosen so that $\eta\{u_k, v_k\} = \theta^{-1}: H_{n_k}(f) \cong \pi_{n_k}(f_{k-1})$.

To obtain a homology decomposition of Y we take X to be a point o. Then $H_r(f) = H_r(Y)$ and X_∞ is a polyhedron having the homotopy type of the singular polyhedron of Y. Moreover, in the sense of a former note,[2] X_∞ is normalized by the subpolyhedra X_k and the k'-classes of the decomposition of f are just the k'-classes of the decomposition[6] of Y. If we take $Y = o$, then $H_r(f) = H_{r-1}(X)$ and we obtain a process for successively killing the homology groups of X.

The *homotopy decomposition* of the map $f: X \to Y$ proceeds in a manner dual to that of the homology decomposition. Propositions 2.2(b) and 2.3(b) replace 2.2(a) and 2.3(a) and the cofibration $j_1: X \to X_1$ induced by the map u is replaced by the fibration $q^1: Y^1 \to Y$ induced by a suitable map $u: Y \to K(\pi_n(f), n)$. We obtain a factorization $f = q^1 f^1$, where q^1 is a fiber map with fiber $K(\pi_n(f), n-1)$ and f^1 is obtained from f by killing $\pi_n(f)$. Iterating the construction, and supposing that in fact $\pi_r(f) = 0$ except for $f = m_1, m_2, \ldots$, where $m_1 < m_2 < \ldots$, we obtain, for each k, a factorization

$$X \xrightarrow{f^k} Y^k \xrightarrow{q^k} \ldots \to Y^2 \xrightarrow{q^2} Y^1 \xrightarrow{q^1} Y, \qquad f = q^1 q^2 \ldots q^k f^k, \tag{3.6*}$$

where the fiber of q^r is $K(\pi_{m_r}(f), m_r - 1)$, $r = 1, \ldots, k$, and $\pi_r(f^k) = 0$, $r < m_{k+1}$, $\pi_r(f_k) = \pi_r(f)$, $r \geq m_{k+1}$. We regard the collection of maps $(f^k, q^k, k = 1, 2, \ldots)$ as a *homotopy decomposition* of the map f. The k-*classes* (Postnikov invariants) of the decomposition are the elements[7] $\{u^k\} \in H^{m_k}(Y^{k-1}, \pi_{m_k}(f))$, where $\{v^k, u^k\} \in H^{m_k}(f^{k-1}; \pi_{m_k}(f))$ was chosen so that $\eta\{v^k, u^k\} = \theta^{-1}: H_{m_k}(f^{k-1}) = \pi_{m_k}(f)$.

If Y is a point then $\pi_r(f) = \pi_{r-1}(X)$ and we obtain the standard Postnikov decomposition of X. Notice that if $\pi_*(X)$ is nonzero in dimensions $p_1, p_2, \ldots$, then the Postnikov invariants are located, as usual, in the groups $H^{p_k+1}(Y^{k-1}, \pi_{p_k}(X))$. If X is a point, we recover the Cartan-Serre-Whitehead technique for killing the homotopy groups of Y.

4. *Remarks.*—(1) We do not claim the homotopy invariance of the homology decomposition. We may prove, however, that if $\Phi: f \to f'$ induces homology iso-

morphisms, then, given a homology decomposition D of f, we may choose a decomposition D' of f' such that Φ respects the decompositions in an evident sense. Thus in particular we may choose compatible decompositions for a cofiber map f and its cofiber F.

(2) Let cat X be the Lusternik-Schnirelmann category of X. Then we may show

THEOREM: *If X is a 1-connected polyhedron with exactly m nonvanishing homology groups, then cat $X \leq m + 1$.*

For X may be regarded as being constructed by successive attachment of cones, and each attachment increases the category by at most 1. It would be interesting to relate the category to the k'-classes. It would also be interesting to dualize the theorem.

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[1] The decomposition is essentially the one described in Moore, J. C., *Séminaire H. Cartan E.N.S.*, 1954–1955, Appendice à Exposé 22.

[2] Eckmann, B., and P. J. Hilton, "Décomposition Homologique d'un Polyèdre Simplement Connexe" (to appear).

[3] Eckmann, B., and P. J. Hilton, *C. R. Acad. Sci. Paris*, **246**, 2555–2558 (1958).

[4] Eckmann, B., and P. J. Hilton, "Homotopy Groups of Maps and Exact Sequences" (to appear).

[5] Eckmann, B., and P. J. Hilton, *C. R. Acad. Sci. Paris*, **246**, 2993–2995 (1958).

[6] We have been informed that E. H. Brown and A. H. Copeland describe a homology decomposition of a 1-connected polyhedron in a forthcoming paper.

[7] The dimensional discrepancies between the descriptions of the homology and homotopy decompositions (e.g., between k'-classes and k-classes) are explained in footnote 2 of reference 5, If they were eliminated the duality aspect of our results would gain, but we would lose the identity between the relative homotopy group of Y mod X and the homotopy group of the inclusion map $X \to Y$. In addition—and perhaps more serious—the Hurewicz homomorphism θ for maps would involve an unfamiliar dimensional displacement.

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*A CHARACTERIZATION OF BIRTH AND DEATH PROCESSES**

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Let $x(t)$ be a stationary Markoff process in a metric space, with transition probability function $P(t, x, E)$. It is a problem of some standing to find conditions expressed in terms of analytic properties of $P(t, x, E)$ which are equivalent to continuity of the path functions. It is known that there are exceedingly pathological processes with continuous path functions. By considering suitable discrete state spaces it is still possible to give a natural meaning to "continuous" path function, and at the same time the fundamental theorems of K. L. Chung[1] show that the pathologies are eliminated if all states are stable. In dealing with continuous state spaces D. Ray[4] and others have found analytic conditions which eliminate the pathologies, and it has been possible to describe large classes of processes whose path functions are continuous in the interior of the space.

The theorem below provides a solution to this problem for a large class of stationary Markoff processes whose state space is the nonnegative integers 0, 1, 2, ... The transition probability function of such a process is an infinite matrix $P(t)$, with entries $P_{ij}(t) = Pr\{x(t+s) = i \mid x(s) = j\}$. A path function of such a process is an integer valued function $x(t)$. It is called "continuous" if it is continuous on the right in the usual sense, and its only discontinuities are simple discontinuities with saltus ± 1. The boundary here is the point at infinity. The path function may go to the boundary in a finite time, but if it does so then it either remains there permanently or else it immediately returns in a continuous way and does not suddenly reappear in a finite state.

In many interesting examples, and in all strongly transient processes, there are permanently absorbing states. Such states do not enter into the Chapman-Kolomogoroff equation in an essential way. For reasons of technical convenience such states will be ignored. This has the effect that a path function $x(t)$ is defined only over an interval $0 \leq t \leq T$ where the positive random variable T is the time of absorption. It also has the effect that perhaps $\sum_j P_{ij}(t) < 1$.

A matrix $(P_{ij}(t))$, $i, j \geq 0$ will be called a *regular Markoff matrix* if

$$(a) \quad P_{ij}(t) \geq 0, \sum_j P_{ij}(t) \leq 1, P(t+s) = P(t)\,P(s), \qquad P_{ij}(t) \to \delta_{ij} \text{ as } t \to 0+,$$

$$(b) \quad \lim_{t \to 0+} \frac{1 - P_{ii}(x)}{t} = q_i, \text{ where } 0 < q_i < \infty.$$

It is known that (a) implies the limit q_i exists and $0 \leq q_i \leq +\infty$, and from (b) it follows that the limits

$$a_{ij} = \lim_{t \to 0+} \frac{1}{t} P_{ij}(t)$$

all exist and are finite.

A matrix $A = (a_{ij})$ will be called a *strict Jacobi matrix* if

$$a_{ij} > 0 \text{ when } |i-j| = 1, \qquad a_{ii} < 0,$$
$$a_{ij} = 0 \text{ when } |i-j| > 1, \qquad \sum_j a_{ij} \leq 0.$$

Thus

$$A = \begin{bmatrix} -c_0 & \lambda_0 & 0 & 0 & 0 & . & . & . \\ \mu_1 & -c_1 & \lambda_1 & 0 & 0 & . & . & . \\ 0 & \mu_2 & -c_2 & \lambda_2 & 0 & . & . & . \\ 0 & 0 & \mu_3 & -c_3 & \lambda_3 & . & . & . \\ . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . \end{bmatrix}$$

where λ_n, μ_n, c_n are all > 0, $\lambda_0 - c_0 \leq 0$, $\lambda_n + \mu_n - c_n \leq 0$.

A regular Markoff matrix $P(t)$ is called *strictly positive of order n* if all of its minors of orders $\leq n$ are strictly positive for every $t > 0$. If $P(t)$ is strictly positive of every finite order then $P(t)$ is called *strictly totally positive*.

With these definitions we have the following

THEOREM. *For a regular Markoff matrix $P(t)$ the following four conditions are equivalent.*

(1) *There is a realization of the process determined by $P(t)$ whose path functions are "continuous" (everywhere, including at ∞). This process has the strong Markoff property and its states form a single communicating class.*

(2) *$P(t)$ is strictly totally positive.*

(3) *$P(t)$ is strictly positive of order two.*

(4) *$A = P'(0)$ is a strict Jacobi matrix and $P'(t) = AP(t) = P(t)A$.*

The proof that (1) implies (2) is contained in reference 3.

That (2) implies (3) is trivial. It is already known (ref. 2, p. 544) that (3) implies $P'(0)$ is a Jacobi matrix, strictness still to come. The backward equation $P' = AP$ is easy to obtain. The key fact is that (3) makes it possible to derive the forward equation $P' = PA$ without resorting to artificial uniformity assumptions. Formally one lets $s \to 0$ in

$$\frac{1}{s}\,[P_{ij}(t+s) - P_{ij}(t)] = \sum_{k \neq j} P_{ik}(t)\,\frac{P_{kj}(s)}{s}.$$

Since $\sum_k P_{ik}(t) = 1$, this formal procedure may be justified by showing $s^{-1}\,P_{kj}(s)$ is uniformly bounded if j is fixed, $k > j+1$ and $0 < s < \epsilon$. The inequality $P_{j+1,\,i}(s)\,P_{k,\,j}(s) < P_{j+1,\,j}(s)\,P_{k,\,i}(s)$ valid if $j+1 < k, j < l$ may be summed for $l \geq j+1$, giving

$$P_{k,\,j}(s) < \frac{P_{j+1,\,j}(s)}{\sum_{l=j+1}^{\infty} P_{j+1,\,l}(s)}\,, \quad k > j+1,$$

and the denominator $\to 1$ as $s \to 0$, which gives the uniformity.

The row sums of A are automatically ≤ 0. If one of the λ_ν say λ_k is zero then the vector $\xi(t) = (P_{k+1,\,0}(t), P_{k+1,\,1}(t), \ldots, P_{k+1,\,k}(t))$ is a solution of $d\xi/dt = \tilde{A}\xi$ where $\tilde{A}$ is formed from the first $k+1$ rows and columns of A. Since $\xi(0) = (0, 0, \ldots, 0)$ then $\xi(t) \equiv 0$ which contradicts $P_{k+1,\,0}(t) > 0$ for $t > 0$. Hence all λ_ν and similarly all μ_ν are > 0.

The proof that (4) implies (1) leans heavily on the results of Chung.[1] We begin by adjoining a permanently absorbing state with index $i = -1$, and define for $i, j \geq 0$

$$P_{i,\,-1}(t) = 1 - \sum_{j=0}^{\infty} P_{ij}(t), \quad P_{-1,\,j}(t) = 0, P_{-1,\,-1}(t) = 1.$$

This gives a slightly enlarged matrix $\tilde{P}(t)$ which is "honest" and which has only stable states. Chung has shown that such a matrix may be realized by a process $y(t, \omega)$ which is separable relative to the closed sets, measurable, whose path functions are continuous on the right, and which has the strong Markoff property. We can assume that for every ω, if $y(\tau, \omega) = -1$ for some $\tau \geq 0$ then $y(t, \omega) = -1$ for all $t \geq \tau$. For each ω let $T(\omega) = \inf\{t;\, y(t, \omega) = -1\}$ and let $x(t, \omega)$ be the function defined only for $0 \leq t < T(\omega)$ and coinciding there with $y(t, \omega)$. We take

$x(t, \omega)$ as a realization of the process determined by $P(t)$, assuming of course that the arbitrary initial distribution for $y(t, \omega)$ is carried by the states $i \geq 0$.

Assume that $Pr\{x(0, \omega) = i\} > 0$ for every $i \geq 0$, and define $\tau_j(\omega) = \inf\{t; x(t, \omega) = j\} \leq +\infty$. Let $\tau_{ij}(\omega)$ be the function whose domain is the ω-set where $x(0, \omega) = i$ and $\tau_j(\omega)$ is finite, and such that $\tau_{ij}(\omega) = \tau_j(\omega)$. Also let $\tau_{kj}^{(i)}(\omega)$ be the function whose domain is the ω-set where $x(0, \omega) = i$ and $\tau_k(\omega) \leq \tau_j(\omega) < \infty$ and such that $\tau_{kj}^{(i)}(\omega) = \tau_{ij}(\omega) - \tau_{ik}(\omega)$. Finally let $F_{ij}(\omega) = Pr\{\tau_{ij}(\omega) \leq t\}$. Because of the strong Markoff property

$$Pr\{\tau_{kj}^{(i)}(\omega) \leq t \mid \tau_{ik}(\omega) < \infty\} = F_{kj}(t).$$

The Laplace transforms $\hat{F}_{ij}(s) = \int_0^\infty e^{-st}\, dF_{ij}(t)$ and $\hat{P}_{ij}(s) = \int_0^\infty e^{-st}\, P_{ij}(t)dt$ are related by the formula $\hat{P}_{ij}(s) = \hat{P}_{jj}(s)\hat{F}_{ij}(s)$ if $i \neq j$. Now the matrix $\hat{P}(s)$ satisfies the equations

$$-I + s\hat{P}(s) = A\hat{P}(s) = \hat{P}(s)A.$$

For the special case when the row sums of A are all zero these equations were analyzed in reference 2, p. 522, and the results show that if $i < j$ then

$$\hat{F}_{ij}(s) = \frac{\hat{P}_{ij}(s)}{\hat{P}_{jj}(s)} = \frac{Q_i(-s)}{Q_j(-s)}$$

where $Q(x) = \{Q_i(x)\}$ is the sequence of polynomials which satisfy $AQ(x) = -xQ(x)$ and $Q_0(x) \equiv 1$. The same method of analysis and the same formula for $\hat{F}_{ij}$ applies in the more general case considered here. It follows that if $i < k < j$ then $\hat{F}_{ij}(s) = \hat{F}_{ik}(s)\ \hat{F}_{kj}(s)$, and that this is also true if $j < k < i$ is a consequence of the symmetry relation $\hat{F}_{ij}(s)\ \hat{P}_{jj}(s)\ \pi_i = \hat{F}_{ji}(s)\ \hat{P}_{ii}(s)\ \pi_j$ where $\pi_0 = 1$, $\pi_n = (\lambda_0\lambda_1, \ldots, \lambda_{n-1})/(\mu_1\mu_2, \ldots, \mu_n)$. From this we deduce that if either $i < k < j$ or $j < k < i$ then

$$\tau_{ik}(\omega) + \tau_{kj}^{(i)}(\omega) = \tau_{ij}(\omega)$$

for almost every ω in the domain of τ_{ij}. Effectively this means that no path can go from i to j without visiting each intermediate state. The above result, taken together with Theorem 4 of reference 1 (in our case all states are stable) leads to the desired implication.

We append a few remarks concerning generalizations and open questions. First it seems perfectly feasible to deal with the case when there is a stable state at ∞—the "extremal solutions of the second kind."[2] It seems likely that the main parts of the argument may be extended to the case of continuous diffusion on a linear interval, although the technical details may here be considerably more difficult. The proof that (4) implies (1) does not involve the notion of total positivity and in principle is not restricted to one-dimensional processes. A purely analytical proof that (4) implies (2) has been given by the authors.[2] Thus the equivalence of (2), (3), and (4) is already established without probabilistic arguments. The theorem itself suggests that there may be a nonprobabilistic proof to the effect that a totally positive semigroup of matrices may be used to define a completely additive measure in the space of *continuous* functions, and the continuous state space analogue of this assertion.

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[1] Chung, K. L., "Foundations of the theory of continuous parameters Markov chains," *Proc. Third Berkeley Symposium*, II, 29–40 (1955).

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[3] Karlin, S., and J. L. McGregor, "Coincidence Probabilities," submitted to *Pacific Journal of Mathematics*.

[4] Ray, D., "Stationary Markov processes with continuous paths," *Trans. Am. Math. Soc.*, **82**, 452–493 (1956).

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*EVIDENCE FOR THE SORTING OUT OF CELLS IN THE DEVELOPMENT OF THE CELLULAR SLIME MOLDS**

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In the development of the cellular slime molds, independent amoebae stream together to central collection points and the resulting sausage-shaped cell masses will migrate for varying periods of time before forming fruiting structures. In a number of species it is evident during the migration phase that the anterior cells of the mass are the cells which will make up the stalk and the posterior cells develop into the sorus, for each individual amoeba in the hind end becomes encapsulated into a spore.

In a study made some years ago Bonner[1] suggested, on the basis of observations with vital dyes, that the position of a cell within the migrating slug was determined by the position of the cell within the aggregation pattern; if a cell were near the center of the aggregating area it would assume an anterior position in the slug, and if a cell were at the periphery of the aggregating area it would assume a posterior position. It is now clear that the method used was totally inadequate and that there is a major rearrangement or sorting out of the cells during aggregation. The evidence for this conclusion comes from a number of previous studies which will be briefly summarized as well as from new experiments that will be presented here.

Before giving the evidence for cell realignment during the aggregation stage, it should be mentioned that there are some observations which indicate a relatively minor rearrangement in the migration stage. Raper[2] was the first to show that if colorless and vitally stained migrating masses of *Dictyostelium discoideum* were cut in two pieces and grafted reciprocally (so that the anterior half was colored and the posterior half colorless, and vice versa) the division line between the colored and colorless regions remained fairly sharp. In repeating these experiments and watching the grafted cell mass for extended periods of time, Bonner[3] showed that a few individual cells did cross the division line and it was even possible to calculate their rate of forward and backward movement.[4] The number of such especially fast- or slow-moving cells was small in comparison to the total number of cells in

the mass and the phenomenon could not account for any major redistribution of the cells within the mass, except over an extended period of time.

In the above experiments the grafts were made in such a way that the normal positions were retained, that is, anterior fractions were kept in an anterior position and posterior fractions in a posterior position. If, on the other hand, a piece of vitally colored anterior region was added to the posterior end of an intact colorless slug, then this colored section slowly moved forward as a unit, while the whole mass migrated, so that ultimately it assumed an anterior position.[3] It was also true that colored posterior grafts placed anteriorly would eventually assume a posterior position. In the controls where posterior bits were placed in the posterior end and anterior bits in the anterior end, the grafts did not shift their positions. Therefore it appeared that the anterior and the posterior cells differed in their rate of movement and if displaced they would return to their original position within the slug. Since normally the majority of the cells within the mass retained their relative positions, there is the possibility that during aggregation the cells have redistributed so that the fast cells are at the anterior end and the slow cells in the posterior end.

To test the possibility of such cell redistribution during aggregation, grafts using amoebae stained with vital dyes were attempted during the aggregation stage.[5] The center of a colorless aggregation pattern was removed and replaced by a vitally stained center (or vice versa) and if there were no redistribution of cells one would expect the resulting slug to be colored at one end and colorless at the other. However the result invariably gave a slug of uniform coloration indicating a major reshuffling while the cells are aggregating.

During the course of the same experiments,[5] it was also shown that the cells of different species and strains had different rates of movement and that the cells of a fast strain could filter past the cells of a slower strain and accumulate in the anterior end of an artificially created chimaera. It is conceivable that the mechanism of cell rearrangement in these strain and species mixtures might parallel the rearrangement within a single strain, which is the subject of this contribution.

The experiments to be presented below were undertaken with two objectives in mind: one was to obtain further and more conclusive evidence whether or not there was normally a sorting out of the cells during aggregation, and the other was to find if any particular character could be associated with the cells that reached the anterior position as opposed to those that assumed the posterior position in the migrating cell mass.

Materials and Methods.—The following slime molds were used for these experiments: *D. discoideum* (DD-1); *D. mucoroides* (DM-11) which includes TYP (or wild type) and two variants of this strain isolated by Filosa[6] which give distinctive abortive fruiting bodies, MV and GV; *Polysphondylium violaceum* (PV-1) which consists of two variants isolated by Filosa,[6] a long and a short migration form. All the slime molds were grown on *Escherichia coli*.

The method for isolating single spores from one sorus was as follows: the sorus was touched with a glass rod possessing a small bead at its tip. The bead was then drawn over the surface of sterile agar in order to spread the spores over a restricted area. Approximately 200 to 250 spores were counted directly on the agar using the high powers of a dissecting microscope (12× oculars, 6× objective). These were

then cut out on an agar block with a small scalpel and placed in 2.0 to 2.5 ml of sterile water (so as to have approximately 100 spores/ml). A loopful of bacteria was added to the tube and after a thorough shaking 0.2 ml was dispensed in each Petri dish of nutrient agar (0.5 per cent peptone, 0.5 per cent dextrose, 2.0 per cent agar). The inoculum drop was then spread over the surface of the agar with a glass rod and the plates were incubated at 18 to 21°C. The growing plaques of amoebae were circled with a glass marking pencil on the bottom of the Petri dish as soon as they appeared and after fruiting their character was recorded and scored.

Results.—Cell redistribution: The basic principle of these experiments was to have a mixture of two cell types in an aggregate and then observe if both cell types were present in equal frequency along the axis of the slug or whether each redistributed itself so that it was predominantly at one end. If the distribution were uneven one could conclude that there had been a rearrangement of the cells, for presumably there was a random distribution of the two cell types over the surface of the substratum before aggregation.

The experiment was done in two ways. In one, a strain which consistently held two cell types in a fairly constant ratio was allowed to aggregate and the emerging sausage was cut into three sections, each producing a small fruiting body. The spores of each of these fruiting bodies were then tested for the relative number of the two cell types by scoring the progeny of the spores upon single spore isolation. When this was done with a mixed strain of *D. mucoroides* (strain DM-11) which contains approximately 85 per cent TYP or wild-type cells and approximately 15 per cent MV cells, then the emerging slug showed 36 per cent MV cells in the posterior fraction (based on counts of 170 spores or plaques, and henceforth the numbers in parentheses will indicate the size of the sample), 6 per cent MV cells (149) in the middle fraction, and 1 per cent MV cells (113) in the anterior fraction.

A similar experiment was performed with *P. violaceum*, but in this case, since the normal fruiting body breaks up into a series of small sori jutting off the main axis of the stalk in whorls, it is not necessary to cut the slug into sections; in fact the cell mass normally breaks up into a series of smaller cell masses from which whorls of side branches simulating miniature fruiting bodies develop. If a strain is used (PV-1) which contains cells which produce colonies that either have a long or a short migration character, then again it is possible to test the distribution of these two cell types within the cell mass. In sori from the posterior end, the frequency of the short migration cell type is 35 per cent (280), while the anterior or apical sorus shows a frequency of 5 per cent (472). (These figures are a summary of five separate experiments all of which showed consistent results.) Since the results for both species show different relative amounts of two cell types along the axis of the slug, there is obviously a redistribution of the cells.

In the second type of experiment the vegetative amoebae of the two cell types (MV and TYP of DM-11) were thoroughly mixed with an eyelash cemented on a glass tube. They were then allowed to continue feeding, and approximately 24 hours after mixing aggregation occurred and the resulting slugs were cut into three sections, again each section producing a small fruiting body, the spores of which were tested. Of two cases examined, one showed 97 per cent MV cells (62) in the posterior fraction, 91 per cent (117) in the middle fraction, and 66 per cent (148) in the anterior fraction. In the second case the posterior fraction showed 86 per

cent MV cells (84), the middle fraction was lost, and the anterior fraction showed 67 per cent (131). These results are consistent with the previous set even though the relative number of MV cells is larger.

Also a few grafts were made at the aggregation stage. If the center of a pure wild-type aggregation pattern was replaced by a pure MV center, one composite slug resulted. This was cut into three sections as before, and in one case the posterior cells were all typical (171), the middle fraction showed 52 per cent MV cells (182), and most important, the anterior fraction had 39 per cent MV cells (153). In other words, the anterior position had changed during the course of the latter half of aggregation from 100 to 39 per cent MV cells, representing a considerable redistribution. This was confirmed in another case (in which the middle and

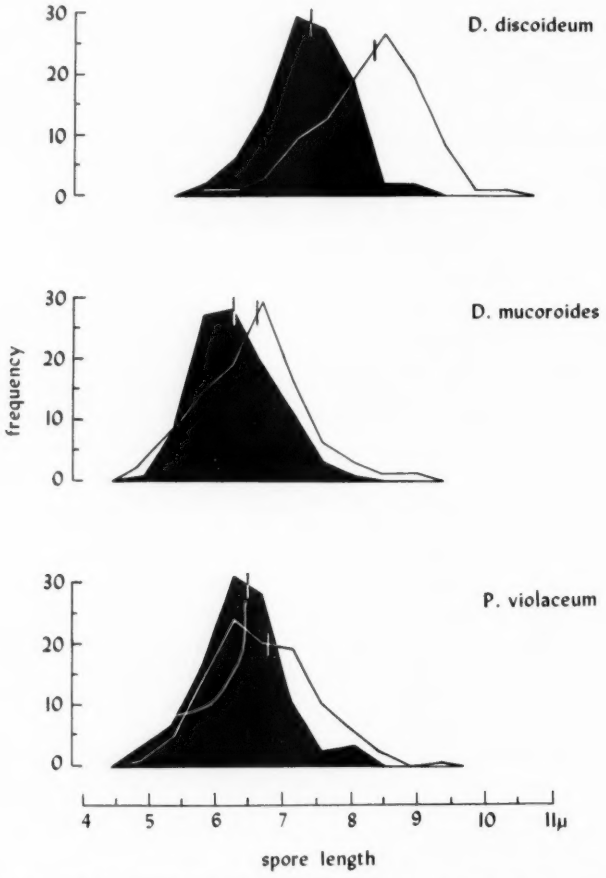

FIG. 1.—Frequency distribution of spore lengths of three species of cellular slime mold showing the difference in size between spores derived from the anterior versus the posterior end of the cell mass. The black curves are of 100 spores derived from the posterior ends, and the white curves are the same for the anterior ends. The vertical markers on the curves show the means.

posterior fractions were lost) where the anterior end went from 100 to 77 per cent MV cells (52).

A Difference between the Anterior and Posterior Cells.—If, as has been demonstrated, there is a redistribution of cells during aggregation, it is now reasonable to ask if any particular character can be associated with those cells that assume the anterior position in contrast to those that assume a posterior position.

The most obvious difference to examine is size. In previous studies it was shown that the anterior cells in the slug of *D. discoideum* are larger than the posterior ones[7] but it was always assumed that this difference did not reflect a difference in the dry weight of the cells, but rather a difference in the amount of water in the cells due to different local physiological conditions in the front and hind end of the slug. To bring the cells to the same physiological condition the following method was used: a slug was cut into three sections and the anterior and posterior fractions were allowed to fruit. Then the size of the spores of the sorus from the anterior fraction was compared with that of the sorus from the posterior fraction. (This was done by drawing the elliptical spores with a camera lucida and measuring their short and long axes. After carefully testing various ways of estimating spore size, it was found that the long axis alone was a completely reliable index of size.)

In this way it was possible to show that in *D. discoideum* spores derived from the anterior end of the slug are significantly larger than those derived from the posterior end. This was done on three separate slugs which showed consistent results and, when combined, gave a total sample of 200 spores for both sori. The mean of the spores derived from the posterior end is 7.3 μ, while that of the spores derived from the anterior end is 8.3 μ, which indicates a clear difference in the size of the population (Fig. 1). (Using the "student" test, the difference gives a t value of 12.4, with a P of > 0.01.)

These measurements were taken from slugs that had migrated approximately 1 cm. By comparison with spores of slugs that had migrated 5 or more cm, it was possible to show that spore size decreases with continued migration. This was interpreted as supporting the assumption that spore size is a reliable index of the dry mass of a cell, for it would be expected that the energy reserves, and therefore the cell dry mass, would be reduced with prolonged migration.

Comparisons of the spores derived from the front and hind end of a slug were also tested on *D. mucoroides* (wild-type clone of strain DM-11). In one experiment, measuring 100 spores for each sorus, it was again shown that the anterior spores were significantly larger. (Difference by the "student" test gives a t value of 3.2, the P being < 0.01.)

Using *P. violaceum*, which has a compound fruiting body, a posterior and an apical sorus were removed and their spore sizes compared. As in the other species, the apical sorus contained spores that were clearly larger. (Difference based on a sample of 100 spores each gives a t value of 3.4 the P being < 0.01).

Discussion.—These experiments support the notion that during the process of aggregation of the cellular slime molds the larger cells sort out so as to assume an anterior position in the migrating cell mass, while the smaller cells assume a posterior position. It would appear that size is directly correlated with speed of movement, although it is possible that selective adhesion might also play a role. It is

hoped that in the near future it will be possible to test further the relation of size to the rate of movement of an individual cell.

If such a sorting out exists during aggregation, there is the interesting corollary that the sorting out itself will produce a gradient of cell size within the mass. The chemotaxis mechanism of aggregation gives a direction or polarity to the cell movement, and since the cells redistribute with respect to size, the inevitable result is a slug possessing a gradient. One could then assume that such a gradient were the basis of the future differentiation of the anterior pre-stalk cells and the posterior pre-spore cells.[5]

Cellular slime molds are sufficiently unusual in so many respects that it always remains doubtful if the lessons we learn from them have any application to other forms. In the development of many organisms there are mass directed morphogenetic movements of cells, however, and it would be of great interest to know if these movements also involve a sorting out of cells which might provide a stepping stone for the subsequent process of differentiation. There is excellent recent evidence of cell sorting in disaggregated tissues, but its role in normal morphogenesis remains virtually unexplored.

Summary.—From previous work there are suggestions that during the aggregation of cellular slime molds there is a redistribution of cells and the more rapidly moving cells assume an anterior position in the resulting slug, while the slower cells lie posteriorly. To test the possibility of cellular redistribution, two compatible cell types were mixed and, although their initial distribution must have been random, their eventual distribution in the slug was markedly non-random. Furthermore, it was found that if a slug were cut up into sections and each fraction allowed to fruit, the spores from anterior fractions were larger than those from posterior fractions. From this it was concluded that normally, during aggregation, there is a sorting out of cells and that the faster-moving large cells are found in higher concentration at the front end of the slug while the smaller cells accumulate at the hind end.

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*AN INHIBITOR OF VIRAL ACTIVITY APPEARING IN INFECTED CELL CULTURES**

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Introduction.—Cytopathogenic viruses may lose their cell-destroying capacity under centain conditions. For example, Type II MEF_1 poliovirus adapted to chick embryo (RMC virus)[1] fails to induce manifest cytopathogenic effect (CPE) in human kidney tissue cells, and multiplies but slightly. In contrast, its capacity under certain conditions to multiply in and destroy human amnion cells is retained.[2, 3] At the outset of this investigation it was noted that CPE of RMC virus in fluids from infected human kidney and amnion cell cultures, in contrast to virus from chick embryos, was markedly inhibited when low dilutions of such fluids were employed as inocula. When the same fluids were further diluted the virus exerted the usual destructive effect on amnion cells. It was thereafter determined that such fluids from human kidney cultures not only inhibited the effect of RMC virus but also certain other viral agents. Experiments here summarized were then undertaken to elucidate this phenomenon.

Materials and Methods.—A stock of RMC virus from the 143rd chick embryo passage and stock strains maintained in this laboratory of polioviruses Type I (Brunhilde) and Type III (Leon), herpes simplex, Sindbis, and vaccinia viruses were employed.

Monolayer cultures were established in test tubes (140 × 15 mm) or Roux bottles with of trypsinized human renal cells which were prepared by Bodian's method[4] and suspended in bovine amniotic fluid medium with 5 per cent normal horse serum (BAF medium).[5] Aliquots of 1 and 50 ml of suspension (3×10^5 cells/ml) were added respectively to tubes and bottles. Cultures were maintained with BAF medium and used experimentally approximately 2 weeks after they were initiated.

For titration and detection of RMC virus, primary cultures of trypsinized human amnion cells prepared according to procedures previously described[6] and nourished with BAF medium were used 2 weeks or longer after initiation and rolled after inoculation. Under these conditions, this virus produces marked CPE. To test the effect on other viruses of "virus-inhibitory fluid" (VIF) containing infective RMC virus, amnion cell cultures about 2 weeks old were left stationary after inoculation. Under these circumstances, RMC virus is not cytopathogenic.[3] In consequence, observed CPE could be confidently attributed to the other virus. All cultures were incubated at 37°C.

VIF was prepared in Roux bottle cultures of human kidney cells containing 50 ml of medium. Approximately 10^5 TCD_{50} RMC virus were added to each bottle. After one day the medium was changed, replaced, and harvested as VIF on the third day. As control for the activity of VIF, fluid was harvested from uninoculated kidney cell cultures prepared with aliquots of the same cell suspension and

thereafter treated in the same manner. This material is referred to as "CF" (control fluid).

Results.—When VIF is titrated in rolled amnion cell cultures moderate to small quantities of RMC virus are demonstrable (Table 1). CPE of this virus in undiluted VIF or in low dilutions of the fluid is, however, completely or almost completely suppressed. To determine whether this inhibitory effect was dependent upon the presence of infective virus or viral antigens and whether this effect was specific in respect to RMC virus or extended to other agents, two types of experiments were done. In the first, VIF was centrifuged at high speed and the supernatant fluid tested for inhibitory effect against RMC virus as well as against polioviruses Types I and III. In the second series of experiments Type II poliovirus antiserum[7] was added in sufficient quantity to VIF to neutralize the virus. The mixture was then tested for inhibitory properties against a number of other viruses.

TABLE I

CPE OF FRACTIONS OF VIF

Dilution of Fraction	Fraction of VIF			Supernatant§ + RMC virus
	Uncentrifuged	Supernatant†	Sediment‡	
Undiluted	0*	0	++++	±
	±	0	++++	
1:2	N.D.	N.D.	N.D.	±
1:4	N.D.	N.D.	N.D.	0
1:8	N.D.	N.D.	N.D.	±
1:16	N.D.	N.D.	N.D.	+
$1:10^1$	++	0	++++	
	++	0	++++	
$1:10^2$	++++	0	++++	
	++++	0	++++	
$1:10^3$	++++	0	0	
	0	0		
$1:10^4$	0	0	0	

0 = no cell destruction.
± = questionable cell destruction.
+ = estimated 25% cell destruction.
++ = estimated 50% cell destruction.
+++ = estimated 75% cell destruction.
++++ = estimated 100% cell destruction.
* = duplicate cultures; 0.1 ml of each fraction was added to a culture. Cultures were observed for 2 weeks after inoculation.
N.D. = not done.
† = obtained by centrifugation in Spinco No. 40 rotor at 104,500 g's for 90 min.
‡ = sediment includes bottom 0.5 ml after centrifugation and button.
§ = amnion cell cultures were overlaid with dilutions of supernatant and each inoculated with 10^3 TCD_{50} stock RMC virus. Cultures overlaid with CF gave ++++ CPE.

From the data in Table 1 it is evident that centrifugation removed all detectable infective RMC virus from VIF. When the virus-free supernatant fluid was added to amnion cell cultures it inhibited CPE of freshly added RMC virus. Furthermore, as shown in Figure 1, CPE of poliovirus Type I was also markedly depressed by the same supernatant fluid. Similar results were obtained with poliovirus Type III.

To 0.8-ml aliquots of VIF, 0.2-ml dilutions of poliovirus Type II monkey antiserum increasing by a factor of 2 were added to a final concentration ranging from 1:5–1:320. A neutralizing antibody titer of 1:360 against 100 TCD_{50} stock RMC virus was found previously for this antiserum. The mixtures were kept at 4°C for 24 hr, and then added to amnion cell cultures after removal of the medium. As controls, a comparable series of mixtures were made employing CF in place of

VIF. After addition of 10^3 TCD_{50} poliovirus Type I to each culture, they were incubated for 8 days. In all controls CPE was minimal at the end of 3 days and maximal after 8 days. In all cultures containing VIF, CPE was absent on the third day and minimal on the eighth day. Similarly VIF with and without antiserum inhibited the action of Types I and III poliovirus in human kidney cell cultures, as well as CPE of Sindbis, vaccinia, and herpes simplex viruses in human amnion or kidney cell cultures in preliminary studies.

Further evidence in support of these observations was obtained by exposing amnion cell cultures to a concentration of stock RMC virus equivalent to that found in VIF. Three hours later, 10^2–10^3 TCD_{50} Type III poliovirus were added and the cultures incubated. The characteristic CPE of Type III virus was not affected, indicating that the presence of such a dose of RMC virus *per se* does not interfere with the course of infection by Type III virus.

Taken as a whole, these results indicate that the inhibitory property of VIF is not dependent upon the presence of infective virus or virus materials that under the experimental conditions can be removed by centrifugation or inactivated by specific antiserum. They also show that the inhibitory effect extends to heterologous strains of poliovirus as well as to other unrelated agents.

Preliminary studies on the mode of action of the inhibitory factor have been carried out. These were aimed at determining whether VIF (*a*) acted directly on the virus, (*b*) decreased adsorption of virus to cells, (*c*) diminished viral multiplication.

(*a*) To aliquots of VIF and CF sufficient poliovirus Type II antiserum was added to yield a final concentration of 1:50. To portions of each mixture an equal volume of poliovirus Type I was added (10^6 TCD_{50}/0.1 ml). After standing at 4°C for 24 hr, dilutions of each mixture were prepared and titrated in amnion cell cultures employing a dilution factor of 3.16 and 3 tubes per dilution. The titers found did not differ significantly. Accordingly VIF does not under these conditions inactivate the virus.

(*b*) Monolayer cultures each containing about 10^5 to 10^6 human kidney cells were exposed either to a mixture of VIF and poliovirus Type II antiserum (final concentration 1:50) or to a mixture of CF and antiserum. The cultures were rolled for 3 hr at 37°C when about 10^6 TCD_{50} poliovirus Type I were added to each. After 1 hr at 37°C the cells were washed five times, treated with poliovirus Type I antiserum, removed from the glass with 0.05 per cent trypsin and counted. Dilutions of the cell suspension were prepared (dilution factor 3.16) and aliquots of each dilution added to amnion cell cultures. The results of the titrations indicated that in both preparations about 1 of 8 cells had absorbed virus. Similar results were obtained using the virus-free supernatant of VIF, without the addition of antiserum. These results, therefore, indicate that VIF does not interfere with adsorption of virus to the cells under the circumstances described.

(*c*) To each of one set of 10 cultures of amnion cells 1 ml VIF freed of detectable RMC virus by centrifugation and to another set 1 ml CF were added after removal of the medium. After standing for 3 hr at 37°C 10^3 TCD_{50} Type I poliovirus were added and the cultures incubated. At the intervals shown in Figure 1 the degree of CPE was recorded and one culture from each set was removed and stored at −20°C. At the end of the fourteenth day the cultures were frozen and thawed

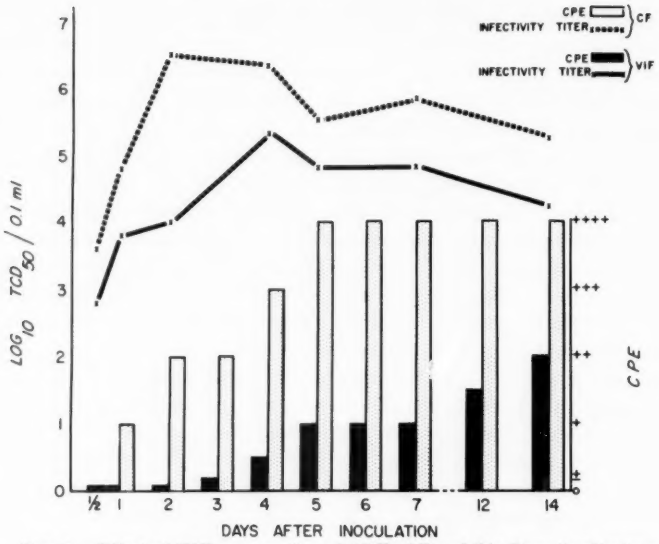

FIG. 1.—Effect of VIF on growth and CPE of Brunhilde Type I poliovirus in human amnion cell cultures. The VIF used was the supernatant fraction which was shown to be free of detectable virus (see Table 1).

three times employing an alcohol-dry ice mixture. The suspensions were then centrifuged at 2,000 rpm for 10 min and the supernatant fluids assayed for infectivity in amnion cell cultures. The efficiency of the release of intracellular virus by freezing and thawing was demonstrated in separate experiments. The results summarized in Figure 1 show that the total yield of virus was consistently lesi in cultures exposed to VIF in which CPE was likewise markedly diminished. Ssnce the experiments were designed to measure the total virus in the two systems at various intervals the findings suggest that in the presence of VIF viral synthesis is reduced. These conclusions were confirmed in another experiment where extracellular and intracellular virus were measured independently.

The data recorded in Figure 1 also suggest that VIF may serve to convert a "virulent" infection characterized by complete destruction of the cell population to a chronic one in which cells continue to survive or multiply and virus to propagate. Additional evidence that VIF may play a role of this sort is provided by experiments in which fluid from HeLa cell cultures chronically infected with RMC virus for over 1 year was found effective in inhibiting CPE of polioviruses Types I and III and Sindbis virus in amnion cell cultures.

Activity of VIF was not removed by dialysis at 4°C against Hanks' solution and BAF medium. It was reduced but not destroyed by heating at 56°C for 30 min, and remained unchanged after repeated (four times) freezing (dry ice bath) and thawing (37°C), and after storage for 1 month at 4°C. Occasionally undiluted CF exhibited a partial inhibitory effect on polioviruses Types I and III that was slightly enhanced in the presence of Type II poliovirus antiserum. Unlike that of VIF the effect of CF was completely eliminated on slight dilution.

Comment.—A factor inhibiting CPE and probably multiplication of several viral strains has been demonstrated in culture fluids of human renal cells infected with a chick embryo adapted strain of Type II poliovirus. The inhibitor can be separated from infective virus and is not inactivated in the presence of homologous antiserum. In certain respects this inhibitor is comparable to "Interferon," a factor appearing in chick embryo tissues exposed to influenza virus.[8] That an inhibitor analogous to that described here may represent an essential determinant in chronic cell infection *in vitro* is suggested by the evidence presented. It is possible that an analogous factor may be produced *in vivo* in areas of infection. If so, it might play a role in the mechanisms of resistance, now largely unknown, that are operative during the acute stage of viral disease.

Summary.—Medium from human kidney tissue culture cells exposed to a strain of avirulent chick embryo-adapted Type II poliovirus inhibits the infection of human amnion and renal cells by homotypic and heterotypic poliovirus as well as other unrelated viruses. This property does not appear to be associated with infective virus or specific viral antigen.

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† Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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SOME STATISTICAL OBSERVATIONS ON A COOPERATIVE STUDY OF HUMAN PULMONARY PATHOLOGY. II

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In our first paper[1] we gave some general average data for the readings of eight pathologists in eight different cities on slides made from sections taken in standard positions in run-of-the-mill lungs at autopsy, using the following classifications: normal, hyperplasia, metaplasia, atypical metaplasia, carcinoma-in-situ and carcinoma. As carcinoma-in-situ was found so rarely by any of the pathologists, that classification will be combined with atypical metaplasia in this continuation of the study; there will be only five groups and their rank indices[2] will be 0, 1. 2, 3, 4.

When we became convinced that the classification was being made on different bases by the different pathologists, we asked all twelve to read a selected sample of 40 slides. This they kindly did, and we reported on the considerable statistical differences in the readings. As the main object in all the work has been to obtain comparable data in the twelve cities for the degree of pathology in the lungs examined, we stated that it would be well to have a considerable sample of the slides from all cities read by several pathologists. The need for this is clear from the differences shown in Table 1 for the percentages of their slides placed in the 5 groups by the pathologists in eight of the twelve cities.[3]

TABLE 1

PERCENTAGE DISTRIBUTIONS FOR MALES, AGE 25 AND UP

Reader	Slides	0	1	2	3	4	Index
J	909	28.8	53.6	11.7	4.2	1.8	0.97
D	941	57.1	21.1	7.7	11.1	3.0	0.82
A	408	38.7	46.1	15.0	0.0	0.2	0.77
E	630	66.7	9.7	18.7	3.6	1.3	0.63
B	223	65.9	9.4	21.1	2.6	0.9	0.63
L	2495	76.4	6.9	11.9	3.3	1.5	0.47
I	669	74.4	8.4	16.3	0.9	0.0	0.44
H	1418	81.8	9.7	8.0	0.4	0.1	0.27
Mean	...	61.2	20.6	13.8	3.3	1.1	0.62

We were fortunate enough to find three of the pathologists who were willing to read a sample of 609 slides drawn from the different cities by random processes.[4] We included also the 40 slides previously read by all twelve. The present paper is a report on the results of the rereading. The two sets of slides will be treated separately. The gross results are in Tables 2 and 3.

TABLE 2

DISTRIBUTION OF TOTAL OF 609 SLIDES ON REREADING

Reader	Slides	0	1	2	3	4	Index
A	609	359	93	127	14	16	0.744
E	609	348	25	212	6	18	0.885
L	609	357	88	133	7	22	0.760
Total	1827	1066	206	472	27	56	0.796

Reader A is high in atypicals (3) and Reader E is low in hyperplasia (1) and high in metaplasia (2) compared with the other two.

TABLE 3

DISTRIBUTION OF THE 40 SLIDES ON REREADING

Reader	Slides	0	1	2	3	4	Index
A	40	4	4	27	3	2	1.875
E	40	5	2	28	2	3	1.900
L	40	5	6	25	1	3	1.775
Total	120	14	12	80	6	8	1.850

In this small sample, distributed very differently from the large one, the differences noticeable in the latter are not in evidence; but the distribution is significantly different from that previously found by all twelve pathologists, viz., 48, 120, 223, 57, 32; though it is not significantly different from what the three rereaders found, viz., 16, 20, 64, 10, 10.

The rereadings of the 40 slides by the three readers and their original readings

have the properties in Table 4. The first reader has not changed his mean significantly, the second has decreased his, and the third increased his, each significantly. The means thus have come closer together. The self-correlation coefficients vary from 0.65 to 0.86.

TABLE 4

Reader	Mean II	Mean I	Mean II − Mean I	Correlation $r_{I,I}$
A	1.875	1.800	$+0.075 \pm 0.114$	0.65
E	1.900	2.125	-0.225 ± 0.103	0.76
L	1.775	1.525	$+0.250 \pm 0.091$	0.86

In the random sample, the numbers of slides belonging to A, E, and L, respectively, were 73, 72, and 60. The comparison of the rereadings by each of his own slides is given in Table 5. It is seen that the three pathologists are reading their own slides about as they did before and that the self-correlation coefficients[5] are of about the same magnitude as for the 40 slides.

TABLE 5

Reader	Mean II	Mean I	Mean II − Mean I	Correlation $r_{II,II}$
A	0.548	0.644	-0.096 ± 0.089	0.60
E	0.792	0.764	$+0.028 \pm 0.073$	0.81
L	1.150	1.333	-0.183 ± 0.142	0.55

With this background we may turn to the standardization of the percentages over classes which result from using the rereadings of the three pathologists as a basis. The method is similar to that on standardizing death rates for age and sex against the age and sex distributions of a standard population. In Table 1, J put 28.8 per cent of his slides in the normals. The sample drawn for J from his 909 slides and presented to the three pathologists among other slides, contained 32 normals, 59 hyperplasias, 17 metaplasias, 5 atypicals, and 3 carcinomas. These were distributed by the three pathologists (averaged) as given in Table 6. We

TABLE 6

Rank	Number	0	1	2	3	4
0	32	31	$^2/_3$	$^1/_3$	0	0
1	59	$38^1/_3$	13	$7^1/_3$	$^1/_3$	0
2	17	3	$^2/_3$	$10^2/_3$	$2^2/_3$	0
3	5	2	$^1/_3$	$1^2/_3$	0	1
4	3	0	0	$^2/_3$	$^1/_3$	2

have to assume that all J's slides of each class would have been distributed in these same proportions had they all been reread. Thus his 28.8 per cent of normals in Table 1 would have been distributed as $^{31}/_{32}$ of 28.8 per cent normals, $^1/_{48}$ of 28.8 per cent hyperplasia, and $^1/_{96}$ of 28.8 per cent metaplasia. In this way one calculates Table 7.

TABLE 7

	0	1	2	3	4
Original	28.8	53.6	11.7	4.2	1.8
0	27.9	0.6	0.3	0.0	0.0
1	34.8	11.8	6.7	0.3	0.0
2	2.1	0.4	7.3	1.8	0.0
3	1.7	0.3	1.4	0.0	0.8
4	0.0	0.0	0.4	0.2	1.2
Standardized	66.6	13.1	16.1	2.3	2.0

The comparison of J's original percentages at the top of this table with the adjustment by the averaged readings of the three pathologists reveals the fact that they would have read his slides very differently and would indeed have given for them a percentage distribution not very far from the mean. This does not mean that J was wrong and they are right; it only means that there is a difference. Treating all eight in the same way, Table 1 as adjusted becomes Table 8.

TABLE 8

ADJUSTED PERCENTAGE DISTRIBUTIONS FOR MALES, AGE 25 AND UP

Reader	Slides	0	1	2	3	4	Index
J	909	66.6	13.1	16.1	2.3	2.0	0.60
D	941	62.7	13.3	18.0	0.8	5.1	0.72
A	408	63.9	15.3	19.9	0.0	0.9	0.59
E	630	68.5	6.5	22.5	1.8	0.8	0.58
B	223	74.3	7.6	14.0	1.0	3.0	0.51
L	2495	60.9	12.2	21.0	0.9	5.0	0.78
I	669	71.1	5.2	20.3	0.5	3.0	0.58
H	1418	68.8	13.3	13.6	0.6	3.7	0.57
Mean	...	67.1	10.8	18.2	1.0	2.9	0.62

When one compares Tables 1 and 8, bearing in mind that, had any three other pathologists reread the slides, the adjustments would have been different,[6] and further bearing in mind that the adjustments have been made by scaling up samples in the different cities of from 60 to 75 with one exceptionally large one of 116, it is obvious that most of the differences between the eight cities have disappeared and that it would be very difficult to separate out from the adjusted percentages items which proved that the pathological conditions of the lungs in the different cities were in fact different.[7]

Even when comparisons of general morbidity or mortality conditions in different places are dubious because of differences in reporting, the analysis of local reports by those familiar with local conditions has value. We hope that the individual pathologists who have been good enough to engage in this survey will work up their data in any way they please. We shall be glad if our study furnishes them something of value for theirs.

[1] These PROCEEDINGS, **43**, 1073–1078, 1957.

[2] This will make the mean indices, standard deviations, and correlation coefficients of the previous paper not strictly comparable with those here, but the comparison will not have to be made.

[3] It has been necessary to omit four of the twelve cities. One of the co-operating pathologists failed to send in the data from his city; one had so few cases that it seemed better not to include his city in the rereading; one sent in no slides to be reread; one had used the Swiss roll instead of the standard sections, and we feared this might introduce noncomparability.

[4] The 609 slides are not strictly random because a few more had been drawn randomly, of which some had to be discarded because at least two of the three rereaders felt that they were not good enough to be read at all. It is, however, our belief that this loss did not seriously disturb the randomness.

[5] The self-correlation coefficients have long been used by psychometrists, educational testers, and others to give one estimate of the reproducibility of the data. See, for example, C. Spearman, *The Abilities of Man, Their Nature and Measurement;* T. L. Kelley, *Crossroads in the Mind of Man: A Study of Differentiable Mental Abilities;* J. P. Guilford, *Psychometric Methods.* On pages 411 ff. of the last is given a brief general discussion of various concepts related to reliability. Our index is a rank index, an index of ordinal position. So are many, if not most, of the grades or marks which teachers use. It may be questionable whether one should treat ranks as cardinal numbers, but that is widely done as we are doing it.

The mean value of the three self correlations on the forty slides is 0.76 ± 0.06, and of those on their own slides is 0.65 ± 0.09. We have six mutual correlations of the three pathologists in pairs on the forty slides and six on their own; the values of the means are respectively 0.69 ± 0.03 and 0.58 ± 0.04. Owing to the small numbers in the samples these means have little statistical stability; but so far as the evidence goes, it indicates that the self correlations are not much larger than the mutual correlations. Or in other words, the three pathologists reproduce one another's readings about as well as they reproduce their own—as measured by these correlations. The natural interpretation is that their remaining differences are chiefly fortuitous or random, due to lack of definition and possibly to lack of complete definability of the pathological material. Some slides may be far from clear; should they be discarded? Some may have part of the mucosae lacking; what about them?

If n, h, m, a, c be the fractions (probabilities) of slides of a certain area on which the worst condition is normal, hyperplasia, metaplasia, atypical metaplasia, and carcinoma, what would be the fractions on slides which had twice that area? This question cannot be answered with any information we have; but it is interesting to consider and may suggest interesting research. If the condition revealed by the slide were so widespread that it would occur on both halves of the slide of double area, there would be no differences in the probabilities. At the other extreme where the (worst) condition is so sharply localised that the condition on the slide had no relation to that on an adjacent equal area, the fractions for slides covering a doubled area could be obtained from combinations of terms in the expansion of $(n + h + m + a + c)^2$. As an illustration, if for slides covering a given area, the fractions (probabilities) are $n = .70$, $h = .10$, $m = .15$, $a = .03$, $c = .02$, then the results for the slides covering twice the area would be 0.49, 0.15, 0.26, 0.06, 0.04, respectively. If the work were to be done over, it might be well to record enough about the conditions appearing on the slides to learn something about their correlations. Such a study might reveal evidence bearing on the question whether in truth the five conditions are in fact successive.

[6] The two cities, J and H, top and bottom of Table 1, which showed the highest and the lowest values of the index and the lowest and highest percentages of normals, were each first adjusted by using the rereadings of each of the three pathologists, and the results were in fact different, as must be expected; but a study of their similarities indicated that an averaging of the findings of the three pathologists should give not only a stabler result but one which would give a standardization worth having.

[7] Consider, for example, what the rereading by A, E, L has done to their own previous distributions:

	A Old	A New	E Old	E New	L Old	L New
Index	0.77	0.59	0.63	0.58	0.47	0.78
Per cent normal	38.7	63.9	66.7	68.5	76.4	60.9

For these three the mean index was 0.62 and has become 0.65—an insignificant change. The differences from the old mean index were +0.15, +0.01, −0.15; from the new −0.06, −0.07, +0.13. Descriptively the correlation is negative, though not significant. The differences from the respective means of per cent normal were −21.9, +6.1, +15.8 and become −0.5, +4.1, −3.5, and again the correlation is negative. This is simply an indication of the differences inherent in passing judgments on the slides. If we correlated the two sets of per cent normal in Tables 1 and 8, we would find $r = 0.24$, and if we correlated the two sets of indices, $r = 0.05$. The striking phenomenon to notice is how much the standardization has reduced scatter.

*BROKEN BOTTLENECKS AND THE FUTURE OF MOLECULAR QUANTUM MECHANICS**

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Dirac once stated that, in principle, the whole of chemistry is implicit in the laws of quantum mechanics.[1] In other words, quantum mechanics offers the possibility that all quantities of chemical interest—the sizes, shapes, and energies of molecules in their ground states and in activated states, and their electric, magnetic, and thermodynamic properties—may eventually be computed purely theoretically. A similar statement applies to the physics and chemistry of solids and liquids.

The present situation for the application of quantum mechanics may be compared with that for the application of the principles of classical mechanics in the years following the discovery of Newton's laws of motion. It took many years for the discovery by Lagrange, Hamilton, and others of the most effective mathematical formulations of Newton's laws. After that, very great and continuing efforts have proved necessary and worth while in using these laws to solve the innumerable physical, astronomical, and engineering problems to which they are relevant. A similar history seems likely, but at an accelerated pace, for the future application of quantum mechanics to chemistry and molecular and solid-state physics. Satellites and space ships will make full use of both classical and quantum mechanics.

There is one factor which tends to obscure the inherently great possibilities for the quantitative application of quantum mechanics to chemical problems. Namely, the activities of tens of thousands of chemists during the last hundred and more years have created an immense body of empirical knowledge, most of which was rather well organized when quantum mechanics arrived in 1925–1926. Quantum mechanics has shown itself extremely fruitful for a qualitative theoretical explanation of, for instance, the empirical rules of valence. But until very recently it has for the most part been less successful in quantitative predictions. We think there are two reasons for this. Namely, first, the answers to the easy problems had already been obtained in the laboratory so that there was no need to ask quantum mechanics to provide them. Second, it so happens that the more difficult problems of chemistry and molecular physics are of a high order of complexity in terms of the mathematical analysis *and* the computational efforts required for quantitative results. However, and this is the point of our paper, those who are working in this field are now catching up, and we believe that the results of theoretical computations are going to compete more and more strongly with experiment from now on.

The present paper deals primarily with quantum-mechanical calculations in the field of molecular electronic structure and chemical valence, but the methods and results should be widely applicable also to solid-state physics and other fields. To understand the present situation better, it will be helpful to look back to the early days of quantum mechanics. For the first few years many of the world's best

theoretical physicists engaged in calculations on molecules using the then new tool of quantum mechanics, in the hope of understanding and explaining molecular properties.[2] Great progress was made in qualitative and semiempirical understanding. The most striking quantitative success of the theory was attained, perhaps, in the computations of Hylleraas on the helium atom and of James and Coolidge on the hydrogen molecule. Here quantum mechanics was shown capable of surpassing experiment in accuracy. But except on the simplest systems the computations proved to be complicated and laborious without yielding more than approximate results. Frustrated and repelled, many of the theorists turned to other problems.

Perhaps the most forbidding difficulty was that of the evaluation and numerical computation of certain integrals representing the energies of repulsion between electrons in different orbits, or as we now call them, orbitals. Even when Heitler and London in 1927 published their well-known paper on the theory of the chemical bond in the hydrogen molecule, they found it necessary to refer their readers to a separate paper by Sugiura for the evaluation of one of the integrals needed.[3] This involved a so-called "exchange integral,"

$$X_{\mathrm{HH}} = \int\int [1s_a(1)1s_b(1)][e^2/r_{12}][1s_a(2)1s_b(2)]dv_1dv_2 \qquad (1)$$

where $1s_a(1)$ refers to electron number 1 in a $1s$ AO (atomic orbital) on atom a, and so on; r_{12} is the distance between electrons 1 and 2, and e is the electronic charge. Yet this integral is only the very simplest example of a type. In the most general integral of Type X, the four $1s$ AOs of equation (1), based on equal nuclear charges, and located on only two atomic centers, are replaced by four AOs which, (*a*) are more complicated in form than $1s$; (*b*) may all be different (e.g., $2s$, $3d\sigma$, $4p\pi$, $5f\delta$); (*c*) may all correspond to different effective nuclear charges; and (*d*) may be located on three or four different centers instead of only on two. Obviously the evaluation and computation in the general case, which is probably needed if real progress is to be made in molecular physics and chemistry, is quite enormously more difficult.[4]

Two somewhat less difficult types of interelectronic repulsion integrals are the two-center "Coulomb" integrals

$$C = \int\int \chi_a(1)\ \chi_a'(1)[e^2/r_{12}]\chi_b''(2)\chi_b'''(2)\ dv_1dv_2 \qquad (2)$$

and the two-center and three-center "hybrid" integrals

$$H = \int\int \chi_a(1)\ \chi_a'(1)[e^2/r_{12}]\ \chi_a''(2)\chi_b'''(2)\ dv_1dv_2, \qquad (3)$$

and

$$H_3 = \int\int \chi_a(1)\chi_a'(1)[e^2/r_{12}]\ \chi_b''(2)\ \chi_c'''(2)dv_1dv_2. \qquad (4)$$

In eqs. (2)–(4), each χ corresponds in general to a different AO, while a, b, c refer to different atomic centers. Besides the two-electron integrals, there are also numerous less difficult one-electron two-center and three-center integrals.

In the integrals just described, the usual AOs are Slater-type orbitals of the form $r^n e^{-\zeta r} Y_{lm}(\theta, \phi)$. More complicated and thereby more accurate AOs and also MOs (molecular orbitals) can be built from these. Hence they and the corresponding integrals appear to be satisfactory building blocks for use in quantum theoretical computations on molecular and solid-state electronic structures. They are equally useful in either of the two principal approaches to the description of such structures, namely the valence-bond and the molecular orbital method.

Since the early years of quantum mechanics, the work of a number of Japanese, English, American, and other investigators has been directed toward breaking the bottleneck of the difficult integrals, but it is only in the last several years that really substantial progress has been made. Among the most active workers have been Kotani and his group in Japan, Boys in Cambridge, Coulson and his group at Oxford, Löwdin and his group in Uppsala, Slater's group at M.I.T., and our own group at Chicago. Computationally practicable expressions for the two-center integrals,[5, 6] and rather less satisfactory ones for the three-center and four-center integrals,[5] were obtained a few years ago.

This was a major advance, and has assisted greatly in making hand or desk computations on molecular electronic structures much more generally feasible than before. An accurate all-electron computation of the wave function of a diatomic hydride (BH), in the SCF-LCAO-MO (self-consistent-field linear-combination-of-AOs molecular orbital) approximation of Roothaan,[7] was published by Sahni in 1956,[8] and a similar all-electron calculation on the nitrogen molecule by Scherr of our Laboratory in 1955.[9] Each of these calculations was for a single interatomic distance, namely the normal equilibrium distance of the molecule, and using the simplest possible orthogonal set of MOs of the kind indicated. More recently, Kotani and co-workers[10] have similarly treated the ground and low excited states of O_2 and Li_2. Duncan at Rochester and H. Kaplan at M.I.T. recently made similar (though more difficult) all-electron calculations on the ammonia molecule, using in part machine calculations.[11] In an earlier all-electron calculation on H_2O by Ellison and Shull, approximate values were used for some of the integrals.

A major and indeed crucial step beyond the development of formulas for molecular integrals is the programming for large electronic digital computers of the otherwise still excessively time-consuming numerical computation of these integrals, and of their combination to obtain the desired molecular wave functions and related molecular properties. The pioneering work in this field was that of Boys at Cambridge, England.[12] Recently, after much preliminary work in this Laboratory, we have developed a successful machine program for (*a*) computing accurately all the necessary integrals for diatomic molecules containing zeroth and first row atoms; (*b*) from these, obtaining SCF-LCAO-MO wave functions and related information.[13] The importance of such a machine program is illustrated by the fact that the entire set of calculations on the N_2 molecule which took Scherr (with the help of two assistants) about a year, can now be repeated in 35 min, using a machine of moderately high capacity (the Remington-Rand 1103 or Univac Scientific, at Wright Field).

For first-row hydride molecules and radicals, e.g., HF or BH, the corresponding time is 10 min, for first-row heteropolar molecules (e.g., CO or LiF), 95 min. The group at Chicago is now engaged in computations on diatomic molecules using this machine program. As a result of these very moderate computation times, which are possible only with a rather large machine, we are in a position to make calculations over a range of interatomic distances, and to vary various parameters so as to obtain better MOs, and in this and other ways to get increasingly good molecular wave functions. Dr. B. J. Ransil has carried out computations of the usual kind on Li_2, N_2, F_2, BH, NH, HF, CO, and LiF, and has gone further by using AOs considerably improved by adjusting their orbital exponents (ζ values). Dr. J. W.

Richardson has used still better AOs in improved calculations on N_2, and much other work toward obtaining the best MOs is under way.

Further, major progress has been made, largely by A. D. McLean, in the efficient programming of 3-center and 4-center integrals. As soon as this work is finished, rapid progress can be made in computations on the structures of triatomic, tetratomic, and larger molecules and radicals, and the way to solid-state applications will also be opened.

As the work continues, increased efficiency and speed of computation and extension to molecules containing second-row and higher-row atoms will be accomplished. Besides the approach we have been following, in which AOs are used in constructing molecular wave functions, very promising machine computations on diatomic molecules are being made by F. E. Harris,[14] using wave functions built from molecular orbitals expressed in terms of elliptic coordinates. Both approaches have distinctive advantages for particular problems. Details of the present and continuing work at Chicago will be published elsewhere.

Looking toward the future, it seems certain that colossal rewards lie ahead from large-scale quantum-mechanical calculations of the structure of matter. As an ideal goal, we might ask for the electronic eigenfunction and energy of every major type of molecule in its ground state and its important excited electronic states, as a function of all possible variations in the nuclear configuration—thereby also including all isomers. We should also like to know how the eigenfunctions and energies vary under the influence of force fields, particularly those resulting from the approach of other molecules. From such information, desired behavior and properties could be deduced by relatively simple calculations.

It can now be predicted with confidence that machine calculations will lead gradually toward a really fundamental quantitative understanding of the rules of valence theory and the exceptions to these; toward a real understanding of the dimensions and detailed structures, force constants, dipole moments, ionization potentials, and other properties of stable molecules and equally of unstable radicals, anions, and cations, and chemical reaction intermediates; toward a basic understanding of activated states in chemical reactions, and of triplet and other excited states which are important in combustion and explosion processes and in photochemistry and in radiation chemistry; also of intermolecular forces; further, of the structure and stability of metals and other solids; of those parts of molecular wave functions which are important in nuclear magnetic resonance, nuclear quadrupole coupling, and other interactions involving electrons and nuclei; and of very many other aspects of the structure of matter which are now understood only qualitatively or semiempirically. From the wave functions of ground and excited states, spectroscopic transition probabilities and lifetimes of excited states can also be computed; these and other items of information which can be obtained for molecules and radicals and are especially difficult to obtain experimentally are important in an understanding of upper atmosphere phenomena and in astrophysics.

The total task of obtaining all these "colossal rewards" from theoretical computations will of course be very large because of their number and variety. It may well be measured in years of fruitful effort by thousands of scientists and their technical assistants, even after fully allowing for the rapid present and future pace of development in the speed and capacity of large-scale computing machines. Ex-

tensive exploration in many directions of the best ways to build atomic and molecular wave functions will first be needed. Because of the nature of the forces acting within molecules and the nature of quantum mechanics, it is unavoidably necessary to proceed step by step by successive (and often quite complicated) approximations. It is doubtful that any simple short cuts can be found, although continually more efficient and rapid methods of approximation may be expected. Really good wave functions, even for simple atoms and molecules, will probably be approached only gradually, though surely.

With each step of improvement, however, there will be new possibilities of insight and correlation. And gradually, reliable computations even of quantities now inaccessible or poorly accessible to experimental observation will come more and more into the picture. Building on these insights and correlations for simpler molecules, taken together with chemical reaction data and spectroscopic and other physical information on larger molecules and solids, it must be possible to arrive at new semiempirical generalizations which should be of great value in understanding and predicting the structure and behavior of large molecules and of solid and liquid aggregates. We think it is no exaggeration to say that the workers in this field are standing on the threshold of a new era. We realize, of course, that ours is only one of a growing community of new eras, but we think that ours is going to be an important member of this community.

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† Slightly revised on the basis of more recent work.

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[14] Harris, F. E., *J. Chem. Phys.*, **27,** 812 (1957); and later work.

*THE HISTORY OF DOUBLE SCATTERING OF ELECTRONS AND EVIDENCE FOR THE POLARIZATION OF BETA RAYS**

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Lee and Yang, in their historic paper[1] suggesting the possibility of nonconservation of parity in weak interactions, pointed out that an experimental test of parity violation must involve the observation of a pseudoscalar. The pseudoscalars they considered in beta decay of nuclei were $(\bar{J}_i \cdot \hat{p}_e)$ and $(\bar{J}_f \cdot \hat{p}_e)$, where $\bar{J}_i$ and $\bar{J}_f$ are the initial and final spins of the nucleus and $\hat{p}_e$ is the momentum of the beta ray. In that paper they concluded that none of the then existing experiments measured either of these quantities. It was later realized[2] that another observable pseudoscalar would be the longitudinal polarization of electrons emitted from unoriented nuclei; i.e., $(\bar{\sigma}_e \cdot \hat{p}_e)$, $\bar{\sigma}_e$ being the spin of the electron. This prediction has been confirmed by a number of experiments.[3, 4]

It is the purpose of this note to point out that in 1928, R. T. Cox, C. G. McIlwraith, and B. Kurrelmeyer[5] carried out an investigation of double scattering of beta rays from radium which can now be interpreted as being evidence for the negative helicity[6] of beta rays and hence evidence for parity violation in weak interactions. The title of their paper is "Apparent Evidence of Polarization in a Beam of Beta Rays." Carl T. Chase[7,8,9] continued the experiments with much improved, more definitive techniques and was able to conclude in his last paper that asymmetry found in the original experiment persisted "not only in every run, but even all the readings in every run, with few exceptions, show the effect." Moreover, he showed that the effect was energy dependent, disappearing when low energy electrons were counted. A search through the literature fails to find any later experiment (prior to 1957) on double scattering of beta rays; all subsequent (again, prior to 1957) experiments[10] were carried out with high voltage electron sources. Before discussing these early experiments we shall briefly review the expected effect of double scattering, first by unpolarized and then by longitudinally polarized electrons. In the examples below the experimental arrangement is that of Cox and Chase; the detector is fixed, the source rotates.

In 1929, Mott[11] showed that the spin orbit coupling in Coulomb scattering could be used as a polarizer or analyzer of electron spin. The spin orbit coupling is largest when the energy of the electrons is relativistic, the Z of the scatterer is high, and large angle scattering occurs; most calculations consider single scattering through 90° by nuclei of $Z \simeq 80$. Mott double scattering is illustrated in Figure 1*A*, where an unpolarized beam of relativistic electrons is first polarized and then analyzed by being scattered singly twice by very thin high Z scatterers; $\hat{n}_1$ is a unit vector in the direction from the source to scatterer 1, $\hat{n}_2$ is a unit vector in the direction of scatterer 2, $\hat{n}_3$ is a unit vector in the direction of the detector. The spin orbit term in the cross section is of the form $\bar{\sigma} \cdot (\hat{n}_1 \times \hat{n}_2)$, so that only a spin perpendicular to the plane of scattering is affected. The single scattered beam at $\hat{n}_2$ will be partially polarized along the $+X$ direction. In the second scattering the spin orbit part of the cross section, $\bar{\sigma}_{+X} \cdot (\hat{n}_2 \times \hat{n}_3)$, will add to the Coulomb

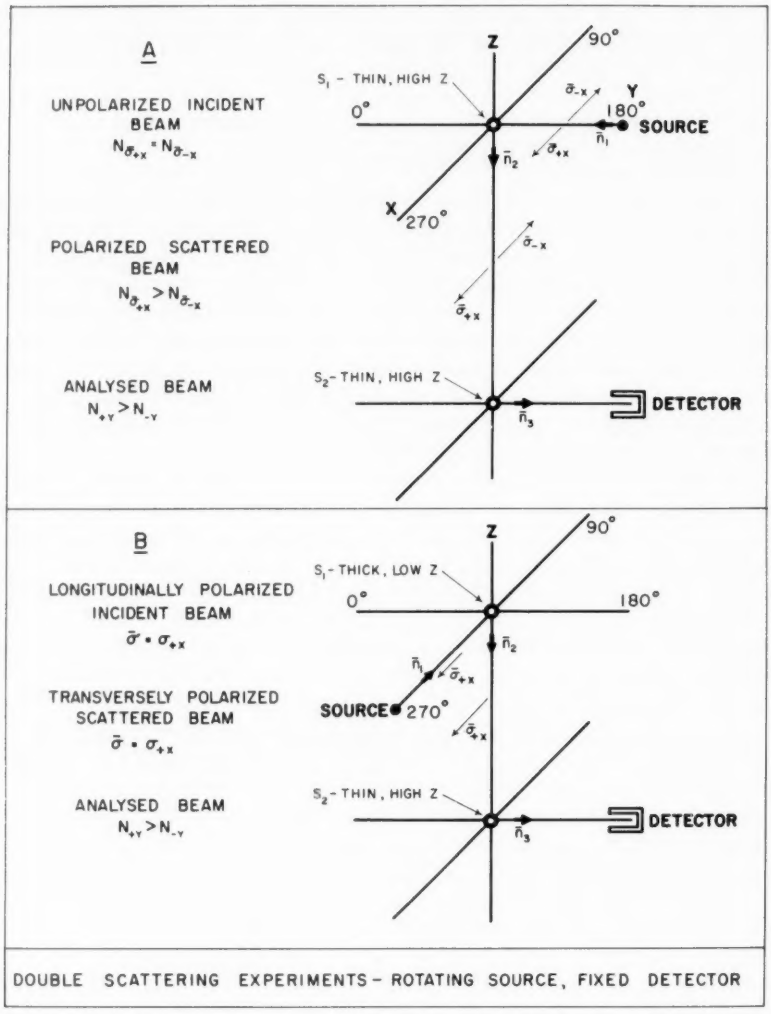

FIG. 1.—*A*, double scattering of an unpolarized beam of electrons. *B*, double scattering of a longitudinally polarized beam of electrons.

term, while $\sigma_{-X} \cdot (\bar{n}_2 \times \bar{n}_3)$ will subtract, so that a greater counting rate will be observed when the source is at the 180° position compared to that observed at the 0° position. No difference in counting rate should be observed when the 90° and 270° positions are compared.

The double scattering of an initially longitudinally polarized beam also results in strong asymmetries. However, the term $\bar{\sigma}_{n_1} (\bar{n}_1 \times \bar{n}_2)$ is zero, so that the asymmetry at 0° and 180° is small and the main effect is observed between the 90° and 270° positions.[12] A double scattering experiment on beta rays in which both

the first and second scatterers are so thin that only single scattering takes place is very difficult. No such experiment has been reported. Experimenters have, however, measured beta helicities by first transforming the polarization from longitudinal to transverse and then utilizing Mott scattering. The transformation may be accomplished by an electric field[3] or by multiple scattering since small angle scattering is spin independent. In the latter case, if the first scatterer is a thick, preferably low Z, material, the spin direction will remain unchanged as the momentum turns and the beam at $\hat{n}_2$ will be transversely polarized in the n_1, n_2 plane, along the X direction in Figure 1, B. When the beam is scattered through 90° by a thin high Z scatterer, the large spin-orbit term will lead to different intensities along the Y axis, or for a fixed detector, a difference in intensities for the source positions at 90° and 270°; in the illustrated example of negative helicity the greater intensity occurs for the 270° position.

The experiment just described—double scattering first by a thick low Z and then by a thin high Z scatterer—has been performed recently by several groups. De Shalit *et al.*[13] measured 6 to 8 per cent asymmetries by scattering electrons of energy greater than 300 kev through 65° by 2.5 mg/cm² thick gold foil (the first scatterer was thick aluminum). Alihanov *et al.*[14] observed asymmetries as large as 40 per cent (and never less than 18 per cent) when 150 to 400 kev beta rays were back scattered through 115° by gold foils ranging from 0.34 to 1.97 mg/cm². They also showed that the asymmetry is essentially independent of the Z of the first scatterer. Heintze[15] obtained 6–12 per cent effects by backscattering ~1 mev betas through 135° by Au and Pt foils.

In the experiment of Cox *et al.* and Chase the beta rays were reflected twice through 90° by thick lead and gold scatterers. The arguments above are then modified in two ways. First, the beam at $\hat{n}_2$ will be slightly depolarized due to plural scattering in the first target (a small effect as Alihanov *et al.* have shown). Second, the observed asymmetries should be much smaller than the recent data quoted above since the multiple scattering in the thick second scatterer is spin independent. However, these same recent experiments argue strongly that a measurable effect should still be observed. In the three previously quoted experiments the second scatterer was so thick, for the angle of scattering used, that the observed effect arose principally from plural scattering (see Cavanaugh[3]). This is especially true in the Alihanov and Heintze experiments where electrons were backscattered from a foil perpendicular to the incident beam. Thus large asymmetries are observed in plural scattering of transversely polarized electrons. Since it is well known that plural scattering is important in scattering from thick targets a measurable effect should be observed in the Cox and Chase experiments. (It is worth noting, too, that Selinger[16] has observed large spin orbit effects by comparing the backscattering of positrons and electrons from thick high Z targets.)

The experiments of Cox *et al.*[5] were initiated by the introduction of the electron spin by Uhlenbeck and Goudsmit.[17] They go on to say "we have been chiefly occupied by an experiment analogous to that in which Barkla demonstrated the polarization of X-rays by double scattering. In our experiment beta particles twice scattered at right angles, enter a Geiger counter. The relative numbers entering are noted, as the angle between the initial and final segments of the path is varied. For reasons to be mentioned later the angles at which most of the obser-

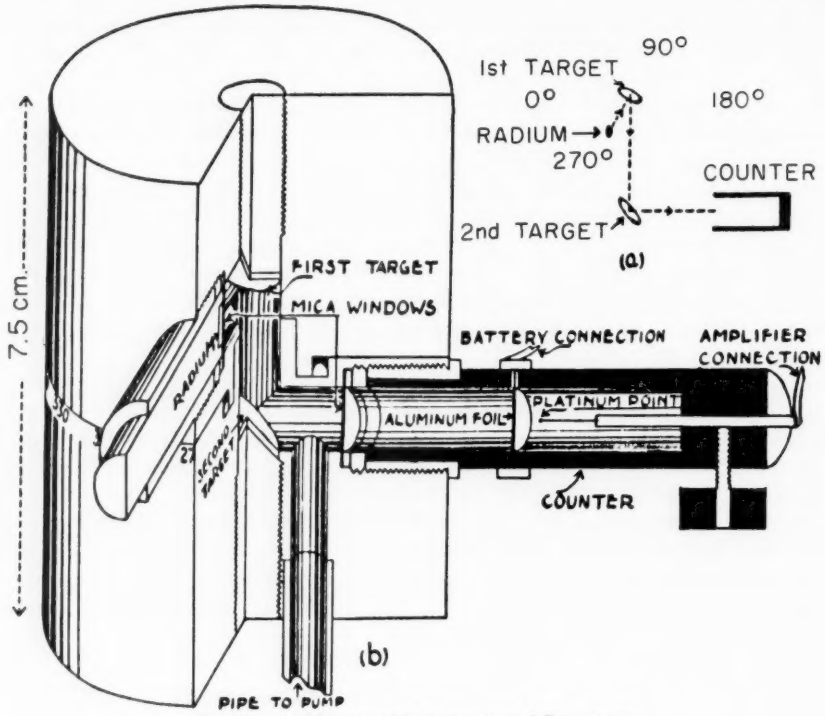

FIG. 2.—Double scattering apparatus of Cox, *et al.*[5]

vations have been made are those indicated in Figure 1 as 270° and 90°. The difference between the configurations of the three segments of path at these two angles is the same as the difference between the right and left-handed rectangular axes. The apparatus in its latest form is shown in detail in Figure 2, a quarter section being supposed removed. . . . Each plug ends in a gold scattering target set opposite one of the radial passages and at 45° to the axes of the two passages . . . . A milligram of radium, which is the source of the beta particles, is set in one of the radial passages and the counter in the other. . . . The cylinder is made in two parts, so that the upper part, carrying the radium and the first target, can be rotated with respect to the lower about their common axis. . . . The cavity is kept evacuated by continuous pumping to a pressure at which the mean free path of a beta particle is large in comparison with the length of its path through the cavity." The Geiger counter was unreliable and had a very short life, of the order of hours, so that the count at one setting was compared with the counts immediately preceding and following it at the other setting. Attention was confined "to taking counts to test an early observation that fewer beta particles were recorded with the radium at 90° than at 270°." A table is presented of 19 sets of data. The average of all of their data, considering the statistical errors only, is $N_{270}/N_{90} = 1.10 \pm 0.01$; the excessively large effect resulting from a few very high values. The authors realize the evident instrumental errors which cause large fluctuations in the data and ascribe

them to malfunctioning of the Geiger counter. They then continue, with the remarkable statement, "A few remarks should be made on the qualitative evidence of asymmetry. Since the apparatus is symmetrical in design as between the two settings at 90° and 270°, the source of the asymmetry must be looked for in an accidental asymmetry in construction or in some asymmetry in the electron itself." Many tests were made on the former point as well as on the effect of the mica windows through which the beta rays passed. They also discuss the effect of a weak magnetic field which may have been present. They conclude that instrumental asymmetries do not account for the effect and furthermore, "it should be remarked of several of these suggested explanations of the observations that their acceptance would offer greater difficulties in accounting for the discrepancies among the different results than would the acceptance of the hypothesis that we have here a true polarization due to the double scattering of asymmetrical electrons. This latter hypothesis seems the most tenable at the present time. . . . It is necessary to suppose further that the polarization is also selective, the effect being manifest only in the faster beta particles. In support of this it may be remarked that a few observations we have just made seem to show that asymmetry is more consistently observed when a piece of celluloid or cellophane is placed in front of the counter to stop the slower beta particles. Perhaps the simplest assumption here is that only beta particles which are scattered without loss of energy show polarization.

"We have made no attempt at a theoretical treatment of double scattering beyond a consideration of the question whether the results here reported are of an asymmetry of higher order than what might be expected of a spinning electron. The following suggestion is then offered not at all as a theory of the phenomenon but merely as a remark on the geometry of the experiment. If it be supposed that the spin vector of a moving electron is always at right angles to its velocity vector and that when the electron is scattered at right angles its new velocity vector has the direction of the vector product of its former velocity and spin vectors and its new spin vector has the direction of its former velocity, then the observations here described will be qualitatively accounted for." The latter explanation, though incorrect, does show that the authors were considering an intrinsic polarization for the electron.

The problem was then taken up by Carl T. Chase, a student of Cox. He improved the apparatus in several essential ways. Path lengths were lengthened, mixed radium was replaced by RaE, lead shields were placed to further reduce gamma ray counts in the counter, provision was made to conveniently block the beta rays and obtain the gamma ray background, and most important of all the counter sensitivity was investigated.[8] Chase showed that the Geiger counters were unreliable for counting relativistic betas and an electroscope was used in place of the Geiger counter.[9] (Chase's apparatus is shown in Figure 3.) This latter change made it very difficult to determine the true errors in counting but did give reproducible data. Chase[8, 9] was able to show that the negative results he obtained in one experiment[7] were due to the low voltage on the Geiger counter which made it insensitive to energetic betas, so that *"with the Geiger counter used as it had been before, at lower voltages, the results were negative as before, but with high voltages on the counter, high enough to ruin the point within an hour or two, the effect was very likely to appear. Making no changes except in the voltage on the counter, the*

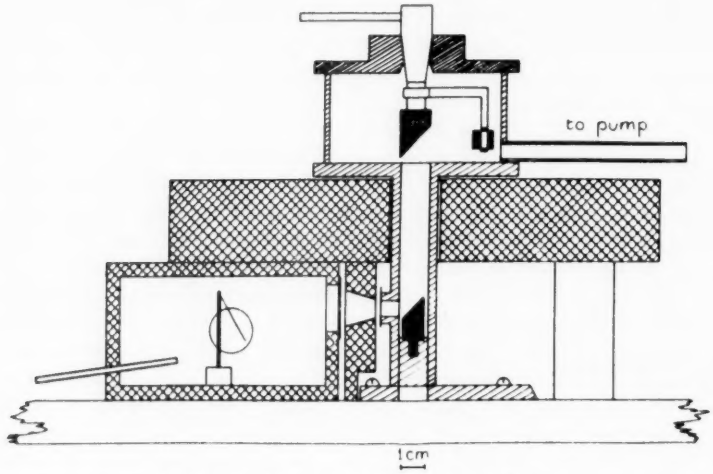

FIG. 3.—Double scattering apparatus of Chase.[9]

effect could be accentuated or suppressed." With the improved apparatus, using an electroscope, he was able to conclude, "the asymmetry between the counts at 90° and 270° is always observed, . . . not only in every single run, but even all the readings in every run, with few exceptions, show the effect." While some difference in counting rate is observed between the 0° and 180° positions, in agreement with the theory of Mott published while the investigation was being conducted, the evident asymmetry in the geometry of the apparatus in these two positions was realized by the experimenters and Chase concludes, "The position of the point at 180° on the curve must therefore be regarded as the least reliable of the set." The effect obtained in this last experiment was $N_{270}/N_{90} = 1.03$.

The objections to these experiments were realized partially by the investigators and in greater degree by subsequent writers. The scatterers were thick so that scattering was multiple and plural and should not show any Mott polarization. The beta rays were uncollimated so that wall scattering was present and the scattering angles were ill defined. The velocity of the beta rays was defined loosely by absorbers and was essentially uncontrollable. For all of these reasons subsequent experiments were done with thin scatterers, better collimation, and electron sources from high voltage accelerators. Experimenters checked their apparatus by showing that no asymmetry existed between 90° and 270°. Nevertheless from the point of view of present understanding these original experiments contained three essential features: (1) The second target was of a high Z material. (2) The geometry was symmetric between 90° and 270°. (3) Relativistic beta rays were used.

It appears in retrospect that these early results were the first experiments showing evidence for the nonconservation of parity in weak interactions.

* Under the auspices of the U.S. Atomic Energy Commission.

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SECRETION AND REABSORPTION IN SWEAT GLANDS

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The foot-pads of the cat are richly supplied with sweat glands of the eccrine type comparable to those that are distributed over the entire surface of the body in man. Sweat glands are blind-ended tubes leading to the skin surface. Each gland consists of a tortuously coiled secreting portion at its blind end and a duct that ends at the surface in a sweat pore. At the transition between secreting portion and duct the tube is particularly thin. Sweat glands are subject to nervous control by the central nervous system and are, in the intact animal or man, more or less constantly active.

Granting the obvious which is that sweat glands secrete, the question whether or or not they, like the kidney tubule, also reabsorb is frequently posed. Direct evidence of reabsorption appears to be lacking. However, reabsorption of water has been postulated by Lobitz and Mason[1] and by Schwartz, Thaysen, and Dole[2] to account for concentration differences between slowly and profusely secreted sweat. Also reabsorption of sodium is proposed by Schwartz and Thaysen[3] to account for sodium concentration differences between blood plasma and sweat. On the other hand, sweat ducts have been regarded as mere channels leading from the secretory portion to the skin surface.

If one were to assume that the ducts of truly resting sweat glands are empty,

then on activation by stimulation of the nerve supply, sweat *formation* in the secreting portion would begin, but a phase of duct filling would antecede sweat *emergence* (which is to say "sweating") at the skin surface. When stimulation is stopped, sweat formation and sweat emergence would cease, but the resting condition would be regained only when the ducts again become empty through reabsorption. Accordingly one might expect observations on the latency of sweat emergence to provide direct evidence concerning the occurrence or nonoccurrence of reabsorption and also to tell something of the dynamics of sweating.

The experiments to be described bring new evidence, of a physical rather than of a chemical nature, to bear upon the question of reabsorption in sweat glands. Study of sweat gland activity in the cat's foot-pad has a distinct advantage in that the secretomotor nerve supply, contained in the plantar nerves, is easily severed to permit the glands to assume the truly resting state and is easily stimulated to induce the active state. In the experiments presently considered, sweat emergence at the skin surface was observed with the aid of a low-power binocular microscope. A stimulator providing variable duration and frequency of electrical stimuli for application to the centrally severed plantar nerves and a stop watch completed the experimental arrangement. The preparations were anesthetized with nembutal, intraperitoneally administered. It is advantageous, in fact virtually imperative, to select cats with black rather than unpigmented foot-pads if one is to see adequately the appearance of sweat droplets. On the black pad, brightly illuminated, they appear as glistening beads upon an otherwise dull background.

After a prolonged rest, something approaching 90 min, stimulation of the secretomotor supply at a frequency of 10 per second, which is maximally effective, may require up to 60 sec duration before the first sweat drops appear. Repetition of the stimulation after a 2-min rest requires but 2.5 to 3 sec. As the duration of rest period is varied between these two extremes, the latency for sweat emergence varies with it in the manner illustrated by Figure 1. Over most of its course the relation

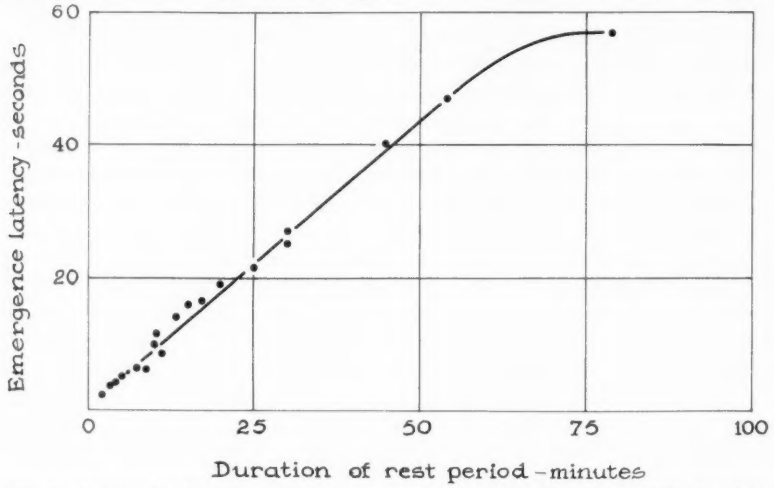

FIG. 1.—Latency for beginning sweat emergence at the surface of the central foot-pad of the cat as a function of rest period duration following a maximal stimulation.

is linear, although the rate of increase in latent period falls off after a rest period of approximately 60 min, and presumably reaches a final value.

When the ducts are full, as they certainly are a very short time after maximal activity, latency for emergence would provide a measure of the latency for sweat formation which on present evidence is some 2.5 to 3 sec. Since the latency for sweat formation would be fixed in fixed conditions of activation, the added time required for sweat emergence with increasing rest period must be time needed for duct filling. Therefore reabsorption must take place in the resting gland. Naturally these experiments do not reveal, except for water, what is reabsorbed by the sweat gland.

The relation between rest period and emergence latency as represented in Figure 1 permits one to make some surmise as to the site of reabsorption in the sweat gland. Since the relation is linear over most its range, it may be supposed that the column of sweat in a duct decrements in length by equal amounts for equal increments in rest period. This presumably would be the case only if reabsorption were to occur near the base of the gland rather than along the length of the duct. Were reabsorption to take place along the entire length of the duct the expected relation between emergence latency and rest period would be logarithmic. Where in the depth of the gland reabsorption occurs remains problematical. Nevertheless it is reasonable to suppose, and the tentative suggestion is, that the initial thin segment of the duct near its junction with the secreting portion may be the locus of reabsorption. The final decline in rate of increase in latent period that occurs at rest periods in excess of approximately 60 min, may indicate that the fluid columns in the ducts of the sweat glands at such times following maximal activity are receding through the region of reabsorption.

In circumstances of maximal activation sweat formation is a rapid process whereas reabsorption is slow. According to the information contained in Figure 1 the relative rates are in the ratio of approximately 75 to 1 and in other experiments the ratio may be as high as 100 to 1. Thus reabsorption cannot be an important determinant of the final product when the glands are maximally active. When minimally active, however, the situation is quite different, as will be seen from experiments of a different sort discussed in connection with Figure 2.

Sweat glands vary in their functional power[4, 5] and it is an important point for consideration that the method of latency measurement here employed yields information concerning the most powerful rather than the average or least powerful sweat glands. One can make a rough visual estimate of the over-all effect of a standard stimulation having, as herein used, a duration of 1 min at 10 per second frequency. Such a stimulation following prolonged rest is not of sufficient duration to secure a full outpouring of sweat. The individual beads indeed may be neither large enough nor sufficiently closely spaced to coalesce. After a brief rest, however, the foot-pad will be drenched long before the end of the stimulation period. In brief, the amount of sweat appearing at the surface may be said to vary roughly inversely with duration of the antecedent rest period.

Unfortunately one cannot extract either from observation of the behavior of the most powerful glands or from observation of the over-all effect of stimulation information as to the exact temporal course of reabsorption in the less powerful glands. If, however, some of the sweat glands, namely those whose reabsorptive activity

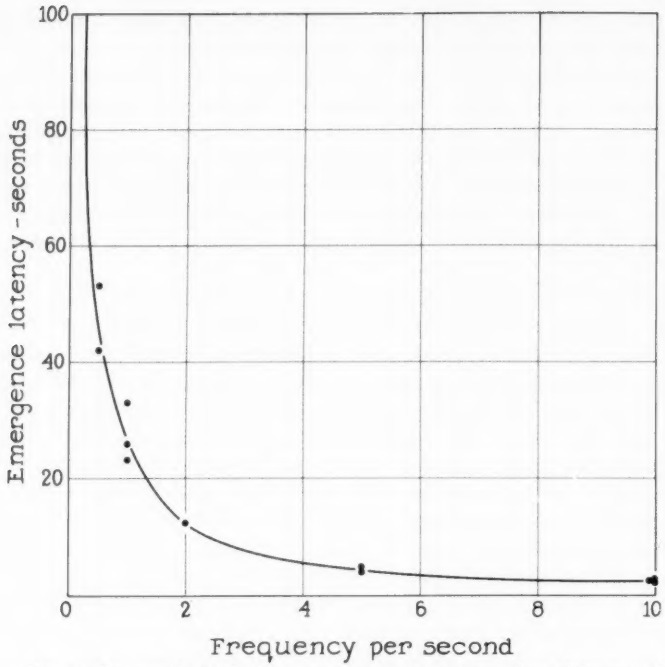

FIG. 2.—Latency for beginning sweat emergence at the surface of the central foot-pad of the cat as a function of stimulation frequency, the sweat ducts having been filled by an antecedent maximal stimulation.

can be measured by available technique, deplete their ducts of sweat content as a linear function of time, then the simplest and most reasonable assumption is that the others do likewise.

Sweating as a Function of Stimulus Frequency.—From the fact that reabsorption occurs in sweat glands it follows that sweating is something that occurs normally when the rate of formation exceeds the rate of reabsorption. In the usual preparation one cannot vary the reabsorption rate but one can, by varying the frequency of stimulation, vary the rate of sweat formation. To observe the behavior of the sweat glands in response to varied frequencies of stimulation, it is a practical convenience to fill the ducts by use of a maximal conditioning stimulation, then to allow a brief rest period of standard duration: 2 min was selected, for at this interval after maximal activation a further maximal stimulation leads to sweat emergence at minimal latency (cf. Fig. 1). In this way standard initial conditions are obtained. A test stimulation of variable frequency then is applied to the secretomotor supply and the latency for sweat emergence observed.

In every instance stimulation at 6 per minute was quite ineffective, although impedance changes in similar circumstances prove the glands active.[6] When stimulated at a frequency of 12 per minute there usually is no visible sweating or, after some 5 to 6 min of stimulation, a few beads may appear. Stimulation at 10 per second induces profuse sweating within 2.5 to 3 sec. As the frequency of stimula-

tion is varied between these two extremes, latency for beginning sweat emergence varies with it in the manner illustrated by Figure 2. At the lower frequencies, latency is quite variable, the flow scanty. With increasing frequency, latency diminishes, becomes more stable, and flow increases progressively.

An interesting fact is that a threshold frequency exists below which sweat formation cannot lead to sweat emergence. In this there is a clear indication that reabsorption takes place in the active gland. For instance, in the experiment illustrated by Figure 2, in the absence of reabsorption one might have expected sweating after 2 min at 12 per minute stimulation frequency and after some 4 min at 6 per minute whereas, in fact, there was none. The threshold stimulation frequency as determined in this way is about 10 per minute, which is to say that the ratio of threshold frequency to maximal frequency is approximately 1 to 60. On the assumption that the sweat formed per secretomotor impulse volley is constant this would indicate that the secretory and reabsorptive actions of the glands come into balance when the secretory action is proceeding at some 1.65 per cent of capacity. Otherwise put, the secretory power of the sweat glands is about 60 times greater than their reabsorptive power, which is in essential agreement with the result discussed in connection with Figure 1.

Again, of course, one must bear in mind the fact that latency measurements as here employed concern the activity of the most powerful glands. As the frequency of test stimulation is lowered from that which is maximally effective, the amount of sweat produced declines. To judge by this fact rather than latency of sweating, the average glands come into secretory-reabsorptive balance at somewhat higher frequencies than do the most powerful glands.

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AVERAGE BEHAVIOR OF SWEAT GLANDS AS INDICATED BY IMPEDANCE CHANGES

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The foot-pad of the cat with its sweat glands may be likened to a thick plate of electrically resistive material with numerous channels bored into its thickness. When these channels are empty the over-all electrical impedance from face to face of the plate would be high but when filled with a highly conductive fluid the over-all impedance would be low. In the foot-pad itself the level of conducting fluid rises or falls within the sweat ducts as sweat secretion or reabsorption predominates.[1] Accordingly there should be, and in fact are, measurable impedance changes associable with secretion and reabsorption. The problems of impedance change in relation to sweat gland activity are numerous and the present account is concerned with but one: its use to secure a better indication than does sweat emergence[1] of the average behavior of sweat glands during secretion and reabsorption. Specifically the influence of varied frequency of stimulation is examined.

To measure impedance Zn-$ZnSO_4$ electrodes are placed one upon the central foot-pad, the other subcutaneously at a short distance. In this way the skin of the cat's foot-pad becomes the major resistive and capacitative component in the unknown arm of an impedance bridge. The bridge, initially in balance with the sweat glands at rest, is thrown progressively off balance as the conducting fluid columns rise in the sweat gland ducts during stimulation and slowly returns to balance as they fall again following the end of stimulation. An example of these impedance changes, oscillographically recorded, is seen in Figure 1.

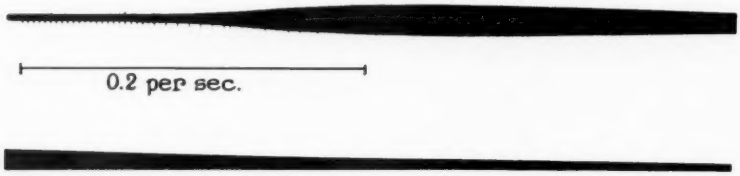

FIG. 1.—Impedance change during and after a stimulation of 5 min duration at 0.2 per second. The upper and lower tracings are continuous. Degree of impedance bridge imbalance is indicated by the width of the line. A-C frequency in this and other figures is 20 cycles. The calibration line indicates 5 min and the time of stimulation.

Although there are many other details, technical and observational, to be considered in a general account of impedance change in relation to secretion and reabsorption[2] the proposition of concern in the present connection is that the impedance level will remain constant in three circumstances only: when the glands are at complete rest, when sweat emergence is maximal and constant during activity, or when the processes of secretion and reabsorption are on the average exactly in balance at some intermediate level of activity.

Figure 2 illustrates the results of a series of 1-min stimulations at a frequency of 10 per second interspersed with 2-min rest periods. Stimulation 2*A* shows the

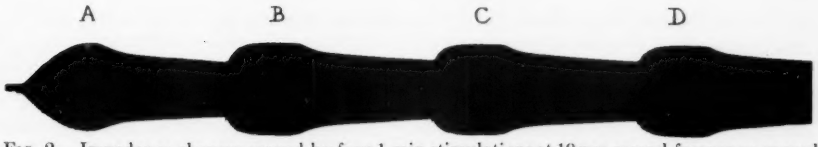

FIG. 2.—Impedance changes caused by four 1-min stimulations at 10 per second frequency spaced 2 min apart. The bridge was originally balanced at rest.

growth from initial resting balance to a certain level of imbalance achieved in 1 min of stimulation following a prolonged rest period. To judge by the steady and constant level of bridge imbalance reached during stimulations 2*B*, 2*C*, and 2*D* a maximal constant level of sweat secretion is reached during those stimulations, each following a 2-min rest period, that is not attained during the first stimulation (2*A*) following prolonged rest. The glands being initially at rest it is known from visual observation[1] that the stimulation 2*A* would have produced a scanty outpouring of sweat after a latency approaching 60 sec, whereas stimulations 2*B*, 2*C*, and 2*D* would have produced profuse sweating early in the stimulation periods.

If a series of stimulations is applied using various frequencies, impedance changes of the sort exemplified by Figure 3 can be recorded. Again the glands initially

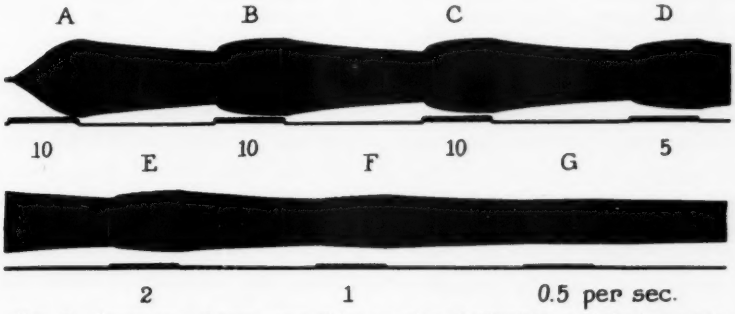

FIG. 3.—Impedance changes caused by a series of stimulations at the frequencies indicated below the stimulus signal tracing. Bridge initially balanced at rest. Upper and lower records continuous.

were at rest and the bridge was balanced. Each stimulation had a duration of 1 min with rest periods of 2 min intercalated between stimulations. Stimulations 3*A*, 3*B*, and 3*C* compare with the first three stimulations depicted in Figure 2 but a clear-cut ceiling on bridge imbalance is not reached in any, and it is not certain from the recordings of bridge imbalance that the ceiling of maximal activity was reached in this preparation in any of these three stimulations. This difference serves to indicate a slight variation between one and another preparation in the stimulation required to produce the same end result.

As stimulus frequency is decreased (3*D*, 3*E*, and 3*F*) increments in bridge imbalance occur during each stimulation but they are progressively less prominent and at a frequency of 0.5 per second (30 per minute), as seen in Figure 2, *G*, the stimulation is only just able to check the decline in bridge imbalance that otherwise would be taking place. There is, therefore, during this last stimulation on the

average a balance established between secretion tending to fill the ducts and reabsorption tending to deplete them.

Figure 4 contains the result of two stimulations, the first (4*A*) at a frequency of 10 per second after a 2-min rest, the second (4*B*) after a further 2-min rest, being a stimulation at 12 per minute for 5 min. In this experiment the low frequency stimulation (4*B*) slows the rate of return of the bridge toward balance but falls just short of checking it. Thus on the average during stimulation 4*B* secretion and reabsorption are just unbalanced in favor of reabsorption.

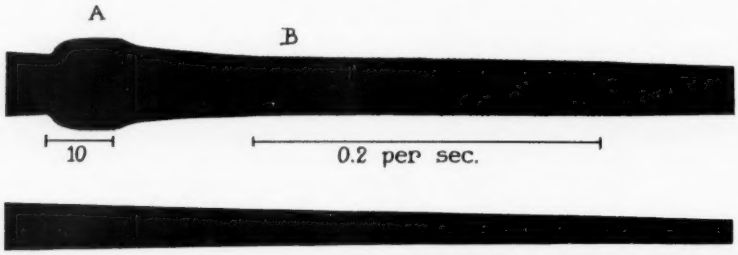

FIG. 4.—Impedance changes caused by a 1-min stimulation at 10 per second (*A*) and a 5-min stimulation at 0.2 per second (*B*) commencing 2 min later. Two minutes before stimulus *A* a like stimulation was applied to the secretomotor nerves. Hence the bridge, initially balanced at rest, is off balance at the beginning of the recording. Upper and lower records continuous.

Although on the average a balance between secretion and reabsorption is struck in those glands that influence the impedance bridge when stimulation frequency is 30 per minute (Fig. 3, *G*), visual inspection in this circumstance of stimulation has never failed to disclose sweat emergence, not copious to be sure, from some of the sweat glands in the microscope field.[1] Stimulus frequency must be reduced to one-third or one-fifth of that which serves for balance in the average gland before the most powerful glands fail to produce some sweat at the surface. This suggests that the most powerful sweat glands are some three to five times more powerful than are the average glands. At the other end of the scale it has been noted that a frequency of 600 per minute is necessary to produce maximal bridge imbalance. Since the ducts of all the glands contributing to recorded change must be full, and all the glands therefore producing sweat at the surface, for bridge imbalance to reach a ceiling the present results indicate that a frequency approaching 600 per minute is necessary for secretion and reabsorption to be just unbalanced in favor of secretion in the least powerful glands. Comparison of this frequency value with that for achieving balance in the average gland (30 per minute) suggests approximately a 20 to 1 ratio for the relative power of the average and of the least powerful glands. Thus, in a crude way, the present experiments indicate for the cat's foot-pad a distribution of variation in functional power of sweat glands not unlike that found by Dole and Thaysen[3] in their precise study of variation in the functional power of human sweat glands.

As an addendum to the main theme of this discussion the present experiments and those of the antecedent paper[1] taken together provide an explanation for certain observed "inconsistencies" in the behavior of "psychogalvanic reflexes." For instance, at high resistance levels skin resistance changes of large amplitude have

been obtained with minimal sweating, and at low resistance levels changes of small amplitude have occurred with considerable sweating.[4] The clue to these findings is that impedance (i.e., resistance) change in action is related to duct filling rather than sweating itself. Thus at the beginning of stimulation 2*A* (Fig. 2) impedance is high; there is a large change during stimulation but, the latency for emergence being nearly as long as the stimulation period, there would be little total moisture produced. On the contrary, at the beginning of stimulation 2*B* impedance is relatively low and the change produced by the stimulation relatively small, but there would be a great outpouring of sweat with minimal latency and hence a large total production of moisture. Now in similar fashion it seems likely that the sweat glands of the human palm that displays a high resistance have been relatively inactive and would exhibit, with little overt sweating, large resistance changes due to duct filling. Conversely, those of the palm that displays low resistance one may suppose have been relatively active and would, their ducts being the fuller, produce copious sweat with little resistance change occurring.

[1] Lloyd, D. P. C., "Secretion and Reabsorption in Sweat Glands," these PROCEEDINGS, **45**, 405–409 (1959).

[2] Lloyd, D. P. C., "Electrical Impedance Changes of the Cat's Foot-pad in Relation to Sweat Secretion and Reabsorption" (in preparation).

[3] Dole, V. P., and J. H. Thaysen, "Variation in the Functional power of Human Sweat Glands," *J. Exper. Med.*, **98**, 129–144 (1953).

[4] Darrow, C. W., "The Significance of Skin Resistance in the Light of Its Relation to the Amount of Perspiration," *J. Gen. Psychol.*, **11**, 451–452 (1934); "The Significance of the Galvanic Skin Reflex in the Light of Its Relation to Quantitative Measurements of Perspiration," *Psychol. Bull.* **31**, 697–698 (1934).

*THE AUDITORY SENSITIVITY OF ORTHOPTERA**

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Recently we reported results obtained by the electrophysiological method on the auditory sensitivity of the Atlantic grasshopper, *Paroxya atlantica* (Scudder) over the range of 100 to 15,000 cps.[1] Though the observation showed that still higher tones were capable of stimulating the tympanal organs of these insects, it was not possible to make quantitative measurements beyond the point indicated because of the limited range of our apparatus. We have now constructed a loudspeaker with a higher range and have extended the calibration of our condenser microphone to 100,000 cycles,[2] and with this improved equipment we have made threshold observations on a variant of this same species of grasshopper, *P. atlantica paroxyoides* (Scudder) and also on some other species of Orthoptera.

The grasshopper used in the present measurements is a somewhat larger form than the one used previously, attaining a body length of about 34 mm in males and 40 mm in females. The procedure was similar to the one used earlier. The insect was deprived of all appendages to reduce its mobility, and an opening was made on the

left side at the level of the first abdominal segment so as to expose the right tympanal nerve. In this exposure a portion of the body wall containing the left tympanal organ was removed, and also a mass of eggs or sperm. The preparation was mounted on a disk of insulating material that could be fitted tightly into the opening of a 1-in. tube leading from a high-frequency loudspeaker. Along one surface of the disk a groove was milled, and at its center this groove was carried all the way through, producing a perforation. The body of the insect was fitted into the groove and plasticene was molded about it to make an airtight seal. The insect was placed so that the tympanic membrane came at the center of the perforation, and thus faced the loudspeaker when the disk was fitted into the sound tube. The dimensions of the disk were chosen so that when it was taken away and the condenser microphone was put in its place the center of the microphone diaphragm was in the same position that had been occupied by the tympanic membrane. Readings of sound pressure were obtained by this substitution method after every series of observations.

A stainless steel microelectrode was applied to the tympanal nerve by means of a micromanipulator, and another electrode was embedded in inactive tissue of the abdomen. The nerve currents produced by acoustic stimulation were amplified and led to a telephone receiver, in which they became audible as sound.

Pure tones were used as stimuli, and were varied in intensity in steps of 5 db to determine a threshold. They covered a range of frequency from 100 cycles upward to the point at which no impulses could be elicited at the highest intensities available. The threshold was definite, and the observations were readily repeatable. Check experiments were made on occasion by exposing the nerve to ether. This exposure produced a prompt cessation of the responses, after which they returned, though with somewhat elevated thresholds, after about 20 min.

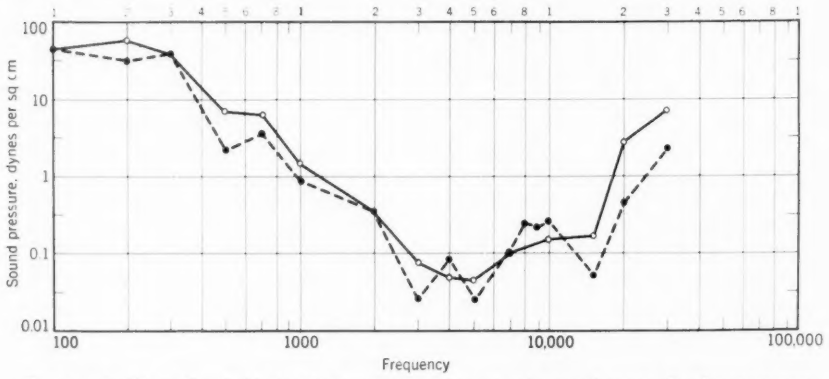

FIG. 1.—Auditory thresholds for the two tympanal organs of a grasshopper, *P. atlantica paroxyoides*. The curves show the minimum sound pressure required to produce impulses in the tympanal nerve.

Results for the two ears of a female grasshopper are shown in Figure 1. They indicate poor sensitivity in the low frequencies, and a progressive improvement as the frequency is raised until a maximum is attained in the region of 5,000 cycles. Then there is a decline in sensitivity until an upper limit is reached at 30,000 cycles.

The two ears show much the same course, and other specimens gave results closely similar to these.

Over the range up to 5,000 cycles these curves resemble the ones obtained earlier for *P. atlantica*, but beyond this point a difference appears. *Paroxya atlantica* was found to continue to gain in sensitivity all the way to 15,000 cycles where the measurements ended, whereas this subspecies shows a continual decline from 5,000 to the upper limit.

Further observations were made on katydids (Tettigoniidae) of two species, *Conocephalus strictus* (Scudder) and *Neoconocephalus retusus* (Scudder), and on crickets (Gryllidae) of the species *Gryllus abbreviatus* (Serville)—the short-winged form of *G. assimilis luctuosus*. For these the procedure was altered somewhat because the tympanal organs are located on the forelegs. A different disk was used, with a hole drilled through its center of a size sufficient to pass the anterior portion of the insect along with the forelegs. As before, the insect was sealed in the opening with plasticene. Also, soft beeswax was used to fix one foreleg in position so that the tympanal surface of the leg was exposed to the sound. The dimensions were such that the tympanal surface was in the same position as the diaphragm of the condenser microphone when the disk was removed and the microphone was in position for calibration. A fine silver wire electrode was inserted through an incision in the upper femur so as to make contact with the tympanal nerve, and another electrode made contact with another region of the leg.

Observations were made on five specimens of *C. strictus*, usually on both forelegs. This is one of the "meadow grasshoppers," inhabiting tall grasses and weedy regions. The males have a length of about 15 mm, and the females one of about 18 mm plus a lance-shaped ovipositor that is longer than the body. Figure 2 shows three of the sensitivity functions, chosen to represent the range of variations found. In these curves we may distinguish three regions, a low-frequency region in which the sensitivity was poor and gained only moderately as the frequency was raised, a middle region of very rapid increase in sensitivity, and a high-fre-

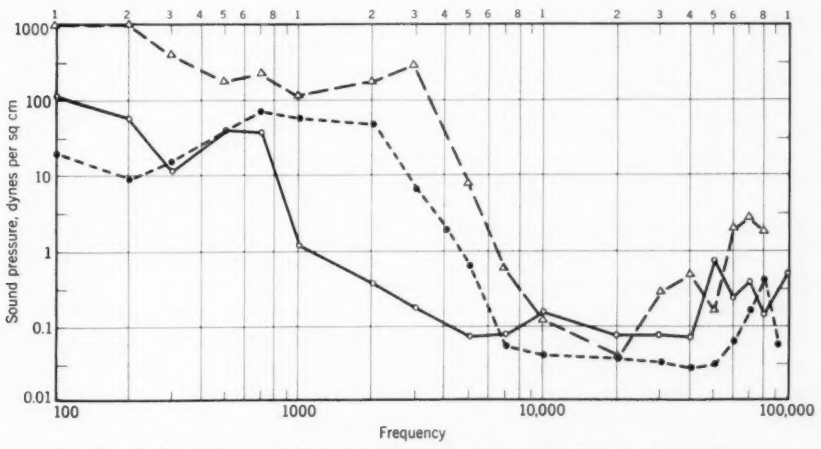

FIG. 2.—Auditory thresholds in three specimens of katydids of the species *C. strictus*.

quency region in which the sensitivity continued at a favorable level for a way and then declined. The most striking variation among specimens was the frequency at which the rapid increase in sensitivity began. This point varied from 700 to 3,000 cycles. At the same time there was a variation in the rate of the increase beyond the breakpoint; the rate was less when the break occurred at a lower frequency and was higher when it occurred at a higher frequency, with the result that when the high-frequency region was reached around 7,000 to 10,000 cycles all the curves were on much the same level. In this region, and up to about 30,000 cycles, the curves for all specimens were fairly similar. Thereafter, in the uppermost frequencies, they showed considerable variations, and showed differences also in the upper limits attained.

Some of the ears ceased to respond (at the available intensities) at frequencies of 80,000 and 90,000 cycles, but most of them continued to 120,000 cycles. We do not have any calibration of our sounds beyond 100,000 cycles, but it is certain that the maximum available pressures declined rapidly from this point onward.

Results obtained from both forelegs of a specimen of *N. retusus* are shown in Figure 3. This is a moderately large species of katydid, and our specimen was a

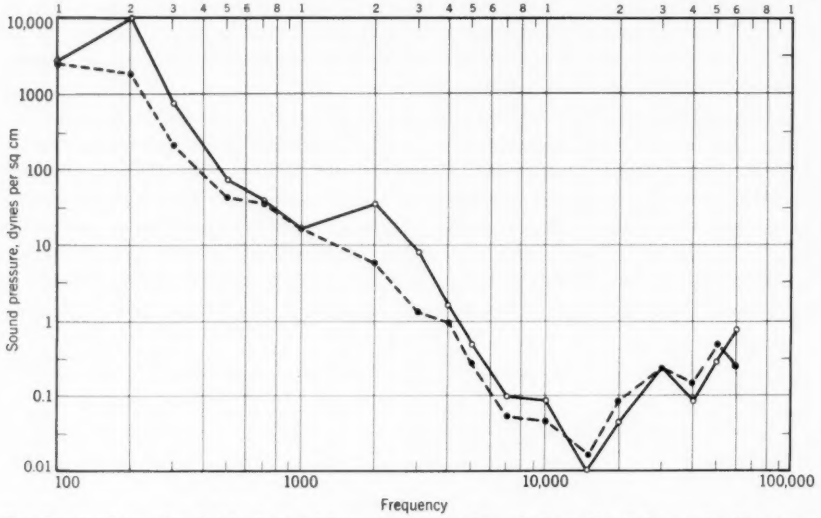

FIG. 3.—Auditory thresholds obtained from the two forelegs of a katydid of the species *N. retusus*.

female, 32 mm in body length. The sensitivity curves show a continuous improvement as the frequency is raised up to 15,000 cycles, and then a decline until an upper limit is reached at 60,000 cycles.

Thresholds obtained from the forelegs of two specimens of crickets (*G. abbreviatus*) are represented by the two lowermost curves of Figure 4. These curves show a progressive improvement of sensitivity as the frequency is raised until 5,000 cycles is reached, after which there is a rapid loss. The upper limit was 15,000 cycles in one of the specimens and 20,000 cycles in the other.

In the fore tibia of the katydids and crickets there are three sense organs, the

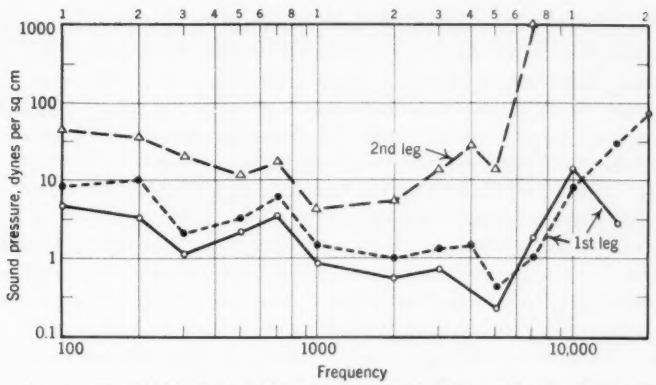

FIG. 4.—Thresholds obtained from three crickets of the species *G. abbreviatus*. The two lower curves represent thresholds obtained from the forelegs, and the upper curve those obtained from the second leg.

crista acustica which is associated with the tympanum, a subgenual organ located just below the tibiofemoral joint, and a small organ lying between these two and called the intermediate organ in katydids and the tracheal organ in crickets. The tibia of the second and third legs contains the last two organs, but lacks the crista acustica with its tympanum.

Figure 5 gives results obtained from one of the forelegs of a katydid (*C. strictus*), and also from one of the second legs of the same insect. Over the range from 100 to 3,000 cycles there is no significant difference between the two legs, but above this range the curves diverge. The curve for the second leg shows a rapid decline in sensitivity and at 10,000 cycles reaches its upper limit. The curve for the first leg, on the contrary, displays its greatest sensitivity in the high frequencies and extends to 90,000 cycles. It seems reasonable to suppose (though the evidence is not conclusive) that the low-frequency region of the functions shown for the forelegs is determined by the subgenual organ, perhaps together with the intermediate organ, whereas the high-frequency region is determined by the tympanal organ.

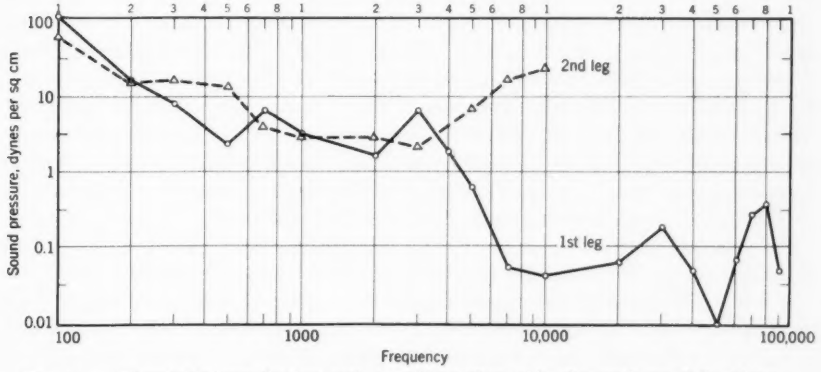

FIG. 5. —Thresholds from the first and second legs of one side of a specimen of *C. strictus*.

This same conclusion was reached by Autrum[3] on the basis of studies carried out on other species of katydids, *Locusta cantans* and *Decticus verrucivorus*. He observed that careful cauterization of the subgenual organ (and probably of the intermediate organ along with it) impaired the sensitivity to low tones but had no effect on that to high tones. On the other hand, destruction of the tympanal organ eliminated the responses to aerial tones above 10,000 cycles but had little effect below 700 cycles. It was his opinion that the tympanal organ is primarily an aerial organ, whereas the subgenual organ is a receptor for mechanical vibrations. He regarded the subgenual organ as responsible for responses to tones below 1,000 cycles and the tympanal organ as most important above 1,000 cycles in the species that he used. In our species the frequency at which there seems to be a transition from one receptor to the other varies in different specimens from 700 to 3,000 cycles.

The uppermost curve of Figure 4 gives results obtained from the second leg of one of our crickets. This curve shows a progressive increase in sensitivity up to 1,000 cycles, and then a decline until an upper limit is reached at 7,000 cycles. The sensitivity is everywhere poorer than that exhibited by the first leg, with the difference varying between 12 and 23 db up to 1,000 cycles and then increasing rapidly to a value of 55 to 60 db at 7,000 cycles.

These results suggest that for the cricket, as for the katydid, the tympanal organ functions most effectively for the high tones, and operates alone in the range above 7,000 cycles. Unlike the katydid, however, the receptors of the foreleg are more sensitive than those of the second leg at the low frequencies also. This means either that the tympanal organ continues to function at the low frequencies or that the subgenual organ of the foreleg is superior to that of the second leg.

The ears of the species of Orthoptera that are considered here show characteristic differences in their patterns of sensitivity. The functions vary in shape, in the maximum of sensitivity and its region of frequency, and in the upper limits attained. The crickets have the poorest hearing. Their limit extends to 15,000 or 20,000 cycles as compared with 30,000 for the grasshoppers and 60,000 or more for the katydids. The crickets have their region of greatest sensitivity at 5,000 cycles, where they are able to respond to sound pressures around 0.2 dyne per sq cm—a level about 1,000 times (60 db) greater than that required by the human ear in its best region around 2,000 cycles. The grasshoppers show the greatest sensitivity in the region of 3,000 to 5,000 cycles, where their threshold is about 0.04 dyne per sq cm—about 46 db poorer than man's at 2,000 cycles. The katydids of the species *C. strictus* are most sensitive over the region from 10,000 to 40,000 cycles, where their threshold also is around 0.04 dyne per sq cm. The specimen of the species *N. retusus* is a little more sensitive, with a sharp peak at 15,000 cycles where the threshold is 0.01 dyne per sq cm, or 34 db worse than man's at 2,000 cycles. Thus in comparison with man—and with any other of the higher vertebrates for which data are available—these insects are not outstanding as regards the maximum of sensitivity attained. Of course we must bear in mind the small size of the tympanal surface, which limits the amount of acoustic energy that can be utilized.

If we make the comparison with the human ear at 15,000 cycles (the highest frequency at which we have human data), then the crickets and grasshoppers still are poorer, and the katydids are of about the same order of sensitivity as man.

* This research was supported in part by the Office of Naval Research, and by Higgins funds allotted to Princeton University. Permission is granted for reproduction and use by the United States Government.

[1] Wever, E. G., and J. A. Vernon, "The Auditory Sensitivity of the Atlantic Grasshopper," these PROCEEDINGS, **43**, 346–348 (1957). Earlier studies of insect hearing by the electrophysiological method are reviewed in this report.

[2] For a fuller description of this apparatus, see Wever, E. G., J. A. Vernon, W. E. Rahm, and W. F. Strother, "Cochlear Potentials in the Cat in Response to High-Frequency Sounds," these PROCEEDINGS, **44**, 1087–1090(1958).

[3] Autrum, H., "Über Gehör und Erschütterungssinn bei Locustiden," *Zeits. f. vergl. Physiol.*, **28**, 580–637 (1941).

DROSOPHILA PAULISTORUM, A CLUSTER OF SPECIES IN STATU NASCENDI, †*

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One hundred years ago Darwin wrote that ". . . species are only strongly marked and permanent varieties, and that each species first existed as a variety . . .." Species of sexual and cross-fertilizing organisms arise mostly through gradual divergence and reproductive isolation of subspecies (= geographic races or varieties). Evidence of this are numerous "borderline cases," in which subspecies have almost reached the degree of divergence and of reproductive isolation met with among species. In turn, the most interesting among "borderline cases" are chains of intergrading subspecies, the terminal links of which coexist sympatrically, in the same territory, with little or no interbreeding taking place. In the territories in which they live together, these divergent forms behave, then, like true species; and yet they appear to be only races if the connecting links are considered. Gene exchange between the terminal links remains possible, and may actually be taking place, via the connecting chain of subspecies.

Several examples of circular chains of subspecies have been observed, chiefly in birds and insects (reviews in Mayr[1] and Rensch[2]). A beautiful case in a species of salamander has recently been added by Stebbins.[3, 4] The first instance in the genus *Drosophila* is to be reported in the present article. According to Patterson and Stone's review,[5] the genus *Drosophila* contained 613 described species in 1952. Several race-species borderline cases have been observed, but in none of these have sympatrically coexisting forms been found connected by chains of allopatric races. Since many species of *Drosophila* are favorable as materials for genetic experimentation, the borderline race-species situation may, it is hoped, be analyzed here more fully than has been possible elsewhere.

Position of Drosophila paulistorum among its Relatives.—The five sibling species, *D. paulistorum*, *D. willistoni*, *D. equinoctialis*, *D. tropicalis*, and *D. insularis* are practically indistinguishable by inspection of their external morphology,[6, 7] although Spassky[8] recently found slight differences in the male genitalia which enable him to classify living males. The species can, however, be identified by

their chromosomes, as seen in the cells of larval salivary glands. Furthermore, they are completely isolated reproductively. Sexual isolation is so strong that cross-insemination rarely succeeds, and no viable hybrids are produced except in the crosses involving *D. insularis*. Even these hybrids are wholly sterile. *D. insularis* is known only from some of the islands in the Lesser Antilles, but the other four species are widely distributed in the American tropics, all of them being sympatric in the basin of the Amazon and in the part of South America lying north of the Equator. *D. paulistorum* occurs from southern Brazil to Central America (Guatemala) and Trinidad. In many parts of this large area it is one of the commonest species of the genus, especially in the superhumid tropical rainforest climates.

D. willistoni is a unified species, in the sense that strains of any geographic origin can easily be crossed with strains from anywhere else, and produce fully viable and fertile hybrids. *D. tropicalis* is differentiated into at least two subspecies, the hybrids between which are sterile as males.[9] Nothing is known about possible differentiation of *D. equinoctialis*. The remarkable differentiation of *D. paulistorum* is described below.

Material.—We have in our laboratory strains of *D. paulistorum* from the following 25 localities (the numbers in the following list correspond to those on the map in Fig. 1): (1) Tikal, Guatemala, May, 1958, Sophie Dobzhansky Coe; (2) Lancetilla,

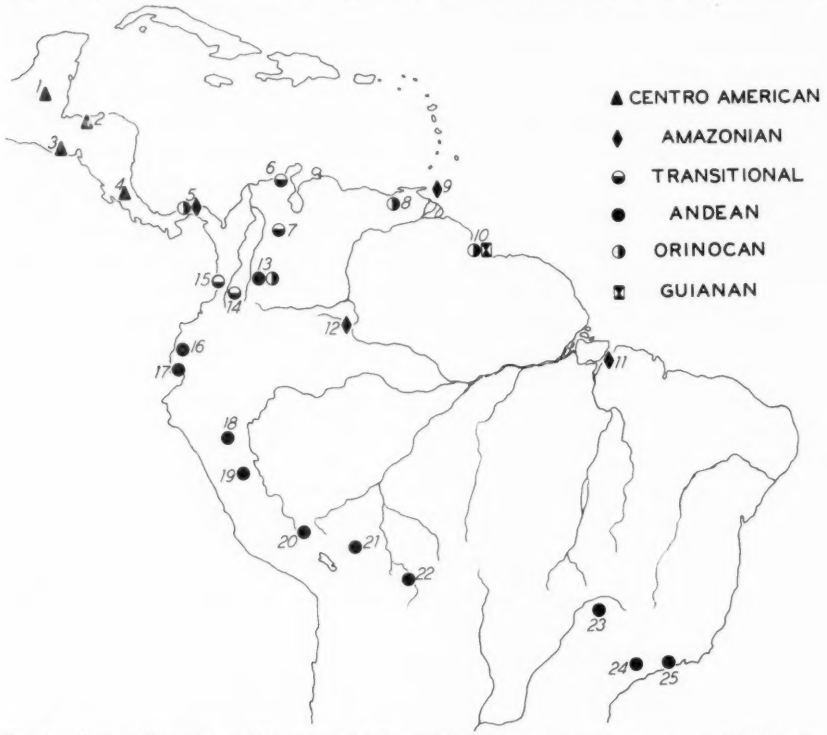

FIG. 1.—Geographic origin of the strains of *Drosophila paulistorum*. The numbers of the localities on this map correspond to those in Figures 2–4 and in the text.

Honduras, May, 1954, W. B. Heed; (3) San Salvador, August, 1954, W. B. Heed; (4) Turrialba, Costa Rica, August, 1956, W. B. Heed; (5) Barro Colorado, Panama, August, 1956, W. H. Heed; (6) Santa Marta, Columbia, September, 1956, H. L. Carson and M. Wasserman; (7) Bucaramanga, Columbia, September, 1956, H. L. Carson and M. Wasserman; (8) Caripe, Venezuela, November, 1956, M. Wasserman; (9) Arima Valley, Trinidad, February, 1956, W. B. Heed; (10) Georgetown, British Guiana, July, 1957, W. B. Heed; (11) Belem, Brazil, July, 1952, Th. Dobzhansky; (12) Içana, Brazil, August, 1952, C. Pavan and Th. Dobzhansky; (13) Llanos near Villavicencio, Columbia, March, 1958, Th. Dobzhansky; (14) Palmira, Colombia, March, 1958, M. Wheeler and Th. Dobzhansky; (15) Buenaventura, Columbia, March, 1958, Th. Dobzhansky; (16) Santo Domingo de los Colorados, Ecuador, March, 1958, M. Wheeler and Th. Dobzhansky; (17) Pichilingue, Ecuador, March, 1958, M. Wheeler and Th. Dobzhansky; (18) Tarapoto, Peru, September, 1956, C. Pavan and Th. Dobzhansky; (19) Tingo Maria, Peru. September, 1956, C. Pavan and Th. Dobzhansky; (20) Urubamba, Peru, September, 1956, C. Pavan and Th. Dobzhansky; (21) Coroico, Bolivia, April, 1958, M. Wasserman; (22) Santa Cruz de la Sierra, Bolivia, April, 1958, M. Wasserman; (23) Ituitaba and Araguari, Minas Gerais, Brazil, August, 1957, M. Breuer; (24) Cantareira, São Paulo, Brazil, January, 1958, C. Pavan; and (25) Angra dos Reis, Brazil, May, 1956, Th. Dobzhansky.

The older strains, the progenitors of which were collected in 1956 or earlier were derived each from a single wild female fertilized in nature. The Peruvian strains (localities Nos. 18–20) are progenies of the populations kept in laboratory population cages for about a year, the founders of which came from several dozen wild strains from a given locality mixed together. The newer strains, started in 1957 and 1958, were prepared as follows. Separate strains were established from each wild female collected in a given locality. These strains were then intercrossed chainwise, i.e., A × B, B × C, . . . Y × Z, Z × A. If all crossed yielded fertile F_1 progenies the strains were pooled in a single mixed population from each locality. Sometimes two groups of strains were found, interfertile within but intersterile between groups. Two pooled strains were then established (i.e., Llanos-A and Llanos-B).

First Series of Experiments.—Attempts to intercross strains of *D. paulistorum* yield a variety of results. Some crosses go easily, and produce fertile F_1 hybrids; others fail to give any hybrid offspring; still others produce in F_1 fertile female but sterile male hybrids. Systematic intercrosses were started in the fall of 1956 and in 1957 with the 16 strains then available. The results are summarized in Figure 2.

The experiments were arranged as follows: about a dozen virgin females from one strain and a like number of males from another were placed together in a culture bottle at room temperature. If no larvae appeared in the food within a week, the flies were transferred, without etherization, to a fresh culture bottle, and left there for another week. If no progeny appeared, the cross was considered as having failed; such failures are marked in Figure 2 by the sign *O*. If the progeny did appear, the hybrid flies were allowed to develop, and a dozen or more pairs of them were placed in a fresh culture. If F_2 larvae appeared, the F_1 hybrids were evidently fertile; such fertile crosses are marked in Figure 2 by an *F*. If no F_2 larvae appeared after a week, the parents were transferred to a fresh culture and left

♂ \ ♀	HONDURAS	SALVADOR-A	SALVADOR-B	COSTA RICA	PANAMA-A	TRINIDAD	BELEM	IÇANA	TARAPOTO	TINGO MARIA	URUBAMBA	ANGRA	BUCARAMANG	SANTA MARTA	CARIPE	PANAMA-B
2. HONDURAS		F		F	O	O	O	O	ST	ST		ST	ST		ST	ST
3. SALVADOR-A	F		F	F	O		O	O	ST		ST			F		ST
3. SALVADOR-B	F	F			O	O	O			ST		ST			ST	F
4. COSTA RICA	F		F		O			O			ST	ST	F	F		ST
5. PANAMA-A	O	O	O	O		F	F	F	ST	O	O	ST	O	O	O	O
9. TRINIDAD		O		O	F		F		O	O	O		O	O		O
11. BELEM	O	O		O	F	F		F	ST		ST			O	O	O
12. IÇANA	ST	O	O		F	F	F		O	O		ST	O		O	ST
18. TARAPOTO	O	ST	O	ST	O		O	O			F	F		ST		O
19. TINGO MARIA		ST		ST		O	O		F			F	F		O	ST
20. URUBAMBA	ST				O	O		O		F		F		ST		ST
25. ANGRA	ST	ST		ST		O	O		F	F			O	ST	ST	
7. BUCARAMANGA		O		ST	O	O	ST		F	F	F	F		ST	O	O
6. SANTA MARTA	F		F		O			ST	ST	ST		ST	F		F	O
8. CARIPE	O	ST	O	ST	O	O	O	ST	ST	O	ST		O	O		F
5 PANAMA-B	ST	ST	ST		O	O	O	O	ST	ST	ST	ST	F	O	F	

FIG. 2.—First series of experiments. Outcome of the crosses between strains of different geographic origin. O—no progeny produced; ST—male F_1 hybrids sterile; F—hydrids fertile.

there for another week. The nonappearance of a progeny was taken to mean that one or both sexes of the F_1 hybrids are sterile. Such crosses are marked in Figure 2 by *ST*. Separate experiments showed that the sterile sex is the male.

The geographic origin of the strains, shown in Figure 1, must be taken into consideration in an analysis of the data reported in Figure 2. A group of "Centro-American" strains are easily intercrossed and yield fertile hybrids; these are the strains from Honduras (the locality No. 2 in Fig. 1), two strains from El Salvador (No. 3), and one from Costa Rica (No. 4). The "Amazonian" group consists of strains from Belem (No. 11), Icana (No. 12), Trinidad (No. 9), and Panama-A (No. 5); these strains also yield fertile hybrids easily. A third, "Andean-South Brazilian," group comes from the eastern slope of the Andes in Peru (localities Nos. 18, 19, and 20), and from Angra dos Reis in southern Brazil (No. 25). The strains of this group also interbreed freely. However, intercrosses of strains belonging to the different groups either fail altogether (*O* in Fig. 2) or yield sterile hybrid males (*ST*).

This situation may suggest the existence of three reproductively isolated, al-

though morphologically identical, sibling species: the Centro-American, the Amazonian, and the Andean. This interpretation is excluded by finding four other strains from Santa Marta (No. 6 in Fig. 1) and Bucaramanga (No. 7) in Colombia, Caripe, in Venezuela (No. 8), and Panama-B (No. 5). Figure 2 shows that Santa Marta gives fertile hybrids with the Centro-American group but not with the Andean group; the Bucaramanga strain is fertile with the Andean group, with Santa Marta, but only with Costa Rica in the Centro-American group. Caripe is fertile with Santa Marta but not with Bucaramanga, and produces sterile hybrids or none with the Centro-American and the Andean groups. Panama-B is fertile with Caripe; its females give fertile hybrids with one of the two strains from El Salvador, and its males do likewise with Bucaramanga. Attempts to cross Panama-B to other strains give sterile hybrids or none.

Perhaps the most important fact is that the two strains, A and B from Panama, have refused to cross entirely, despite repeated trials. Since both strains are derived from progenitors collected in Barro Colorado Island, this locality must harbor two sympatric, and yet completely reproductively isolated, populations of *D. paulistorum*. These populations behave, then, like full-fledged species. And yet they can exchange genes, because each of them can be crossed to other populations with which they produce at least fertile female hybrids. In fact, crosses can be arranged connecting the two Panama populations without yielding any sterile hybrids at all (see below).

The matings which produce no hybrids at all (marked by an *O* in Fig. 2) fail because of a complete sexual isolation between the strains involved. This has been established by dissection of the surviving females which were exposed for two weeks to foreign males, and examination of their seminal receptacles under a microscope. No sperm was found in the hundreds of females dissected. There is an incomplete sexual isolation also between at least some of the strains which produce sterile hybrids (*ST* in Fig. 2). We have dissected the females from some of these crosses after the hybrids have appeared, and found usually one or two inseminated females, the rest remaining virgins. This is evidently the explanation of the erratic character of some of the results; thus, the females of the Amazonian strains always refuse to accept males of the Centro-American and Andean groups, but Amazonian males occasionally inseminate some females of the latter two groups. Perhaps, any two strains may eventually be made to produce hybrids. Whether any of the inter-group crosses yielding sterile hybrids are entirely free of sexual isolation remains to be determined.

The nature of the sterility of the hybrid males is also not known in detail. It can, however, be stated that the testes of these males contain no spermatozoa and only abnormal spermatids, and their seminal vesicles are always empty.

Second Series of Experiments.—The experiments reported above made it clear that the species *D. paulistorum* is broken up into at least three incipient species, plus some connecting, or transitional, populations. In 1958 one of us (Th. D.) was able to collect further material in Colombia and in Ecuador. Drs. M. Wheeler and M. Wassermann of the University of Texas have contributed invaluable material from Panama, British Guiana, and Bolivia; Drs. C. Pavan and A. B. da Cunha have sent new strains from southern Brazil. A second series of experimental crosses has been carried out in 1958, using the same procedure as described above,

♂ \ ♀	GUATEMALA	HONDURAS	COSTA RICA	PANAMA-A	TRINIDAD	BELEM	PANAMA-B	PANAMA-C	LLANOS-A	CARIPE	GUIANA-A	GUIANA-B	SANTA MARTA	BUCARAMANGA	PALMIRA	BUENAVENTURA
1. GUATEMALA		F	F	O	ST			ST	ST	ST	ST	O	F	O		ST
2. HONDURAS	F		F	O	O			ST		ST			F		O	
4. COSTA RICA	F					O	ST		ST		O	ST		O		O
5. PANAMA-A	O		O		F		O		O		O			ST	O	O
9. TRINIDAD			O			F	O	O	O		O	O		O	O	O
11. BELEM	O	O		F	F			O		O			O		F	
5. PANAMA-B	ST	ST				O		F	F	F	F		ST	O	O	ST
5. PANAMA-C		ST	ST	O	ST		F		F	F	F			O	O	ST
13. LLANOS-A	ST	F				ST	ST	ST		ST	O	O	F	O	O	
8. CARIPE			ST	O	O		F	F	ST		F		ST		F	ST
10. GUIANA-A	O	ST		O		O		F	F	F		O	ST		O	
10. GUIANA-B	ST	ST		O	O			O	ST	O	O		ST		ST	
6. SANTA MARTA			F	O	O			O			O			F	O	F
7. BUCARAMANGA	O	ST				ST		ST	O	ST	O	ST				
14. PALMIRA			F	O	O		O		O					F		ST
15. BUENAVENTURA	ST	ST				O		ST	ST	ST	ST	O	F		O	
13. LLANOS-B	ST	ST		ST		ST	ST	O	O	ST	ST	ST	ST	F		
16. SANTO DOMINGO	F		O	O	ST		ST		F					F		ST
17. PICHILINGUE	ST	ST				O		ST	ST	ST	ST	O	F	ST	F	ST
19. TINGO MARIA	ST	ST				O		O		ST	ST	ST	ST		F	
21. COROICO			ST	O	O		O		O					F		F
22. SANTA CRUZ	ST	ST				ST		O		O	ST	F	ST		F	
23. MINAS GERAIS			ST	ST	O		ST		ST							F
24. SÃO PAULO	ST	ST				O		ST		ST	ST	O	ST	F	F	
25. ANGRA			ST	O	O		O		ST					F		F

FIG. 3.—Second series of experiments. Outcome of the crosses between strains of different geographic origin. O—no progeny produced; ST—male F_1 hybrids sterile; F—hybrids sterile.

except that no dissections and examinations of the sperm receptacles were made. The results are summarized in Figures 3 and 4, and in map form in Figure 1.

(*a*) *Centro-American group,* Guatemala to Costa Rica. Fully fertile *inter se* and with Santa Marta, Colombia. The strain from Tikal, Guatemala, collected

♂ \ ♀	SANTA MARTA	BUCARAMANGA	PALMIRA	BUENAVENTURA	LLANOS-B	SANTO DOMINGO	PICHILINGUE	TINGO MARIA	COROICO	SANTA CRUZ	MINAS GERAIS	SÃO PAULO	ANGRA
1. GUATEMALA	F	O		ST		ST	ST	ST	ST		ST		F
2. HONDURAS	F		O			ST		ST	F		ST	ST	ST
4. COSTA RICA		O		O	ST		O	ST	ST	ST		ST	ST
5. PANAMA-A		ST	O	O	O		O	ST		ST		ST	
9. TRINIDAD		O	O	O	O		O	ST		ST		ST	
10. BELEM	O		F			ST			ST		ST		ST
5. PANAMA-B	ST	O	O	ST	ST		O	ST		O		ST	
5 PANAMA-C		O	O	ST		ST			ST		ST		ST
13. LLANOS-A	F	O	O	O	O	ST	ST	ST		ST	ST	ST	ST
8. CARIPE	ST	O	F	ST		ST		O	ST		ST		ST
10. GUIANA-A	ST		O			ST			ST	ST	ST		ST
10. GUIANA-B	ST		ST			ST		ST	ST	ST	ST	O	ST
6. SANTA MARTA		F	O	F		ST	F		O		ST		ST
7. BUCARAMANGA					F		O	F		F		F	
14. PALMIRA		F		ST	F	F			F		F		F
15. BUENAVENTURA	F		O		ST		F	ST		ST		F	
13. LLANOS-B	ST	F	F			F	O	F		F		F	
SANTO DOMINGO		F		ST	F				F		F		F
17. PICHILINGUE	F	ST	F	ST		F		ST		ST		ST	
19. TINGO MARIA	ST		F			F			F		F		F
21. COROICO		F		F	F		F				F		F
22. SANTA CRUZ	ST		F			F		F	F			F	
23. MINAS GERAIS				F	F		F			F			F
24. SÃO PAULO	ST	F	F			F		F	F		F		
25. ANGRA		F		F	F		F			F		F	

FIG. 4.—Second series of experiments. Outcome of the crosses between strains of different geographic origin. O—no progeny produced; ST—male F_1 hybrids sterile; F—hybrids fertile.

by Mrs. Sophie Dobzhansky Coe, yields fertile hybrids also with Santo Domingo, Ecuador. The strain from Costa Rica is fertile with Palmira, Colombia.

(*b*) *Amazonian group*, Panama to Para. Fertile *inter se*, and with only one other strain, from Palmira, Columbia (Transitional group, No. 14). Amazonian

females accept males chiefly from the Andean group, but amazonian males are more successful with females of all other groups.

(*c*) *Andean-South Brazilian group*, Llanos of Colombia, Andes of Ecuador, Peru, and Bolivia, southern Brazil. There is some incipient differentiation even within this group, since the males from Pichilingue, Ecuador, yield sterile F_1 hybrid sons when mated to Peruvian, Bolivian, or Brazilian females (Fig. 4). Pichilingue females produce fertile hybrids with Peruvian, Bolivian, and Brazilian males. Otherwise this group gives fertile hybrids only with some of the strains of the Group F (Transitional), but sterile ones with other strains of the Transitional and other groups.

(*d*) *Orinocan group*, Panama, Llanos and Colombia, Venezuela, British Guiana. Fertile *inter se*, except that Llanos-A males produce sterile sons with females of the other strains, and Llanos-A females produce sterile sons with males from Caripe, Venezuela. The Llanos-A strain is also fertile with Honduras of the Centro-American groups, with Santo Domingo of the Andean group, and with Santa Marta of the Transitional group.

(*e*) *Guianan*, a single strain, Guiana-B from near Georgetown, British Guiana. Thus far, we have succeeded in obtaining fertile hybrids between this strain and only one other, that of the Andean group, from Santa Cruz, Bolivia. Guiana-B males are accepted by females of the other groups except the Amazonian, but the male hybrids produced are sterile.

(*f*) *Transitional group*, Colombia localities Nos. 6, 7, 14, and 15 (Fig. 1). Fertile *inter se*, except that the strains from Palmira and from Buenaventura are difficult to cross, and when crossed produce sterile sons. This group deserves its name because at least some of its members produce fertile hybrids with at least some members of every other group, except the Guianan group. Here we have, then, a genetic bridge which connects the gene pools of the other groups. A glance at the map in Figure 1 shows that Group F is geographically also bridging the other groups.

Conclusions and Summary.—The geographic distributions of the six groups, or subspecies are, as shown in Figure 1, generally distinct. However, in at least three localities representatives of two groups occur together, sympatrically, and yet remain incapable of interbreeding. These localities are: (1) Barro Colorado Island, Panama, where groups B and D have been found, (2) Llanos near Villavicencio, Colombia, groups C and D, and (3) Georgetown, British Guiana, groups D and E. In all these cases the sympatric populations yield no hybrid progenies, and even no cross-insemination, in laboratory experiments in which they have no possibility of mating within their own race.

Inability of sympatric Mendelian populations to interbreed and to exchange genes is *prima facie* evidence that these populations belong to different species. Nevertheless, we are obliged to conclude that *D. paulistorum* is, considered as a whole, a single species. This is not because its component populations, whether reproductively isolated or not, are not distinguishable by the morphology. The compelling evidence comes from the existence of bridging populations, which do produce fertile hybrids when crossed to other populations which are reproductively isolated from each other. With the strains at our disposal, it may be necessary to make as many as four consecutive crosses in order to connect two otherwise non-interbreeding populations without encountering sterility of, at least, the male

hybrids (e.g. between Panama-A and Panama-B). Since female hybrids are mostly fertile, the gene exchange becomes facilitated; generally no more than two consecutive crosses are needed to open a channel for gene exchange. *D. paulistorum* is a single inclusive Mendelian population. No matter to what group a strain giving rise to a favorable mutation or a gene combination may belong, the mutation of the gene combination will, at least potentially, be able to diffuse in other populations and to become the property of the species as a whole. To what extent such a potentiality is actually realized is, of course, a separate question. We hope to be able to shed some light on it by another study.

Comparison of *D. paulistorum* with other race-species borderline case, that of the frog *Rana pipiens*, suggests itself. As shown by the brilliant studies of Moore,[10] the northern populations of this species (from New England) yield inviable hybrids when crossed to southern populations (Texas or Florida). Viable hybrids are, however, formed by geographically adjacent populations. *R. pipiens* is an inclusive Mendelian population, a single species, and, at least potentially, a unit of evolutionary change. One can imagine the situation in *R. pipiens* coming to resemble that in *D. paulistorum*, if the frog populations like those of New England migrated to some territory and met there expanding populations like those of Texas, and both coexisted sympatrically without effective gene exchange. If this were to happen together with preservation of the present genetic connection in the eastern United States, we would be led to conclude that the specific unity of *R. pipiens* is not forfeited. On the other hand, suppose that only populations like the present inhabitants of New England and Texas were preserved and became sympatric without breakdown of reproductive isolation; that would mean emergence of two genetically closed systems, two species. A parallel event would be disappearance of the populations of *D. paulistorum* which now inhabit the northwestern part of the continent of South America (Colombia and, perhaps, Panama). What remained would, perhaps, be five independent species (here, however, a caveat: the populations of central and eastern Brazil having not been studied, we do not know whether the Amazonian and the Andean groups are, or are not, connected there).

Whether two Mendelian populations, or groups of populations, are distinct species or only races is frequently in doubt. Most of these doubtful cases can be resolved unambiguously if sufficient evidence, observational and experimental, becomes available. A minority are, however, true borderline cases; in these the process of species splitting has, on our time level, reached the critical stage of transition from race to species. *D. paulistorum* is one species; it is also a cluster of species *in statu nascendi;* it bespeaks the correctness of Darwin's opinion that "each species first existed as a variety."

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